Transgene Insertion of Cathelicidin Gene in Channel Catfish *Ictalurus punctatus* using CRISPR/Cas9 Knock-in Technology and Cathelicidin Activity Against Catfish Pathogens

by

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Abstract

Protection of fish against infectious diseases is a major challenge in aquaculture, and economic losses due to these diseases limit profitability. Antimicrobial peptides (AMPs), a class of highly conserved peptides known to possess direct antimicrobial activities against invading pathogens, were evaluated for their ability to protect channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) against infection caused by the catfish pathogens, *Aeromonas hydrophila* ML09-119 and *Edwardsiella ictaluri* S97-773. To identify effective peptides, the minimum inhibitory concentrations (MICs) against pathogens namely *Edwardsiella ictaluri* S97-773, *E. piscicida* E22-10, *A. hydrophila* ML09-119, *A. veronii* 03X03876 and *Flavobacterium columnare* GL-001 were determined *in vitro*. Cathelicidins derived from alligator and sea snake exhibited more potent and rapid antimicrobial activities against the tested catfish pathogens when compared to cecropin and pleurocidin AMPs, and ampicillin, the antibiotic control. When the peptides (50 µg/ml) were injected into fish and simultaneously challenged with each pathogen through immersion, increased survival rates in channel and hybrid catfish were observed for both cathelicidins (alligator and sea snake) as compared to other peptides and the infected control. Bacterial numbers were also reduced in the liver and kidney of channel catfish and hybrid catfish in the cathelicidin treatments 24 h post-infection. After 8 days of *E. ictaluri* challenge, serum was collected to determine immune-related parameters such as bactericidal activity, lysozyme, serum protein, albumin and globulin. These immune-related parameters were significantly and consistently elevated in fish injected with the two cathelicidins as compared to other peptides and the infected control. These results show the potential of cathelicidin to protect catfish against bacterial infections and suggests an

approach that overexpressing the peptide in transgenic fish may provide a method of decreasing bacterial disease problems in catfish.

To produce disease-resistant lines of channel catfish carrying cathelicidin gene, targeted gene insertion using CRISPR/Cas9 knock-in system was used. Two types of donor vectors, dsDNA and plasmid DNA, were designed, which were driven by two different promoters, zebrafish ubiquitin promoter and common carp β-actin promoter, harboring a 250-bp homologous sequences flanking both sides of the genomic target locus in a non-coding region of channel catfish chromosome 1. High integration rates were observed using dsDNA and plasmid DNA construct driven by zebrafish ubiquitin promoter. However, upon analysis, integration rates were higher in dead fry than in live fingerlings, indicating either off-target effects or pleiotropic effects. Additionally, we may be targeting a sensitive area of the genome. Furthermore, low levels of mosaicism were detected in the tissues of P1 individuals harboring the transgene, and high transgene expression was observed in the blood of some P1 fish. This can be an indication of localization of cathelicidin in neutrophils and macrophage granules as also observed in most antimicrobial peptides. Overall, these findings prove a successful targeted exogenous gene insertion in a non-coding chromosomal region in non-model fish using CRISPR/Cas9 knock-in system.

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List of Abbreviations

AMP	Antimicrobial Peptide
BHIB	Brain Heart Infusion Broth
BPI	Bactericidal Permeability-Increasing Protein
CAMP	Cationic Antimicrobial Peptide
Cas9	CRISPR-Associated Protein 9
CEME	Cecropin-Melittin Hybrid Peptide
CFU	Colony Forming Unit
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DSB	Double-Strand Break
dsDNA	Double-Stranded Deoxyribonucleic Acid
ESC	Enteric Septicemia of Catfish
gRNA	Guide RNA
HDP	Host Defense Peptide
HDR	Homology Directed Repair
HR	Homologous Recombination
IHNV	Infectious Hematopoietic Necrosis Virus
IL	Interleukin
LPS	Lipopolysaccharides
MAS	Motile *Aeromonas* Septicemia
MIC	Minimum Inhibitory Concentration
NHEJ	Non-Homologous End Joining

OD	Optical Density
PAM	Protospacer Adjacent Motif
PBS	Phosphate Buffered Saline
QPCR	Quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
RBL	Rhamnose-Binding Lectin
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
sgRNA	Small Guide RNA
ssDNA	Single-Stranded Deoxyribonucleic Acid
TALEN	Transcriptional Activator-like Effector Nucleases
TICAM 1	Toll/Interleukin 1 Receptor Domain-Containing Adapter Molecule 1
TLR	Toll-like Receptor
TSB	Tryptic Soy Broth
UTR	Untranslated Region
ZFN	Zinc Finger Nucleases

CHAPTER ONE

General Introduction

Catfish are an important food fish in the United States, but are susceptible to several infectious diseases, which constitute the primary limiting factors of catfish production. About 45 percent of inventory losses on catfish fingerling farms are attributable to infectious bacterial diseases (MSU, 2012). Disease outbreaks can reduce profitability by increasing treatment costs, decreasing production, reducing feed conversion efficiency and causing harvest delays. Efforts to reduce such losses include effective fish health management, use of antibiotics and development of vaccines. However, a consequence of the use of antibiotics in aquaculture is the presence of drug residues which could lead to the development of bacterial resistance and even toxicity to consumers (Okocha et al., 2018). Vaccines, on the other hand, have some drawbacks because aside from being expensive and laborious, vaccination can involve stressful handling of the animals. Furthermore, vaccines are not available for all of the fish pathogens and some existing vaccines do not perform well (Assefa and Abunna, 2018).

An alternative strategy to control diseases in aquaculture would be to develop disease-resistant genotypes. Genetic improvement of disease resistance traits based on traditional breeding programs may be time-consuming and frequently, the outcome is unpredictable as some desired genetic traits have low heritability. In the past three decades, gene transfer technologies have been explored and numerous transgenic fish developed, making transgenesis a promising method to produce a superior fish for the future. Substantial research has focused on genetic improvement of growth rate in aquaculture species (Rahman and MacLean, 1999; Cook et al., 2000; Khalil et al. 2017; Kishimoto et al. 2018), but significant advancement has also been

accomplished in the enhancement of disease resistance (Dunham et al., 2002; Mao et al., 2004; Chiou et al., 2014). Here, the role of exogenous peptides with antimicrobial properties in transgenic fish as well as the targeted gene insertion using CRISPR/Cas9 gene-editing technology are reviewed and discussed.

Antimicrobial peptides in transgenic fish

Antimicrobial peptides (AMPs) are naturally occurring polypeptide sequences present in virtually all species and composed of cationic and hydrophobic amino acids with broad spectrum antimicrobial properties (Hancock and Sahl, 2006; Haney et al., 2019). Well-known examples of cationic antimicrobial peptides with broad-spectrum antimicrobial activity *in vitro* and *in vivo* are the cecropins, defensins, lactoferrins, hepcidins, melittins, pleurocidins and cathelicidins (Hancock and Lehrer, 1998; Yamauchi et al., 1993; Hsieh et al., 2010; Pan et al., 2011).

Gene transfer of antimicrobial peptide genes may improve bacterial resistance in fish up to three- to four-fold (Dunham, 2009). In model fish such as medaka (*Oryzias latipes*), Sarmasik et al. (2002) demonstrated that F2 generation expressing cecropin transgenes had acquired elevated resistance to bacterial infection. Challenge studies revealed that approximately 10% of medaka transgenic individuals were killed by *Pseudomonas fluorescens* and about 10-30% by *Vibrio anguillarum* as compared to 40% of the control fish that were killed by both pathogens. Moreover, hepcidin, an AMP derived from tilapia (*Oreochromis mossambicus*), were overexpressed as a transgene in zebrafish (*Danio rerio*) and convict cichlid (*Archocentrus nigrofasciatus*), and the F3 generation had increased resistance to *Vibrio vulnificus* infection and various immune-related gene expressions were altered after different bacterial infections (Hsieh et al., 2010). Recently, another AMP identified in Nile tilapia *Oreochromis niloticus*, tilapia piscidin (TP3), was introduced as plasmid DNA in zebrafish embryos at the one-cell stage (Su et

al., 2018). When compared to wild type fish during a bacterial challenge with *Streptococcus agalactiae*, F2 progeny of transgenic zebrafish exhibited higher resistance to the pathogen *S. agalactiae* and elevated expression of Toll-like receptor 4a (TLR4a), interleukin (IL)-10, IL-22, and C3b. Transgenic manipulations of antimicrobial peptide genes in these studies demonstrated that disease-resistant fish lines could be produced not only in model fish species but also in aquaculture species.

Exogenous peptide expression in non-model fish

Effective peptides have been inserted into the genomes of some economically important fish to produce an innately disease-resistant species that can confer immunity against pathogens. Cecropin, which was first discovered in the hemolymph of a moth *Hyalophora cecropia*, was an effective AMP that was shown to have positive bactericidal activity to fish pathogens such as *Pseudomonas fluorescens*, *Aeromonas hydrophila* and *Vibrio anguillarum* (Sarmasik and Chen, 2003; Chiou et al., 2006). Cecropin B construct driven by the CMV promoter was inserted into channel catfish *Ictalurus punctatus* and challenge results showed enhanced resistance to bacterial diseases such as columnaris and enteric septicemia of catfish (ESC) by two- to four-fold (Dunham et al., 2002). Chiou et al. (2014) also used the same cecropin B construct to produce homozygous transgenic rainbow trout, *Oncorhynchus mykiss*. Challenge studies showed that many families of F2 and F3 transgenic rainbow trout expressing cecropin B exhibited resistance to infection by *Aeromonas salmonicida* and infectious hematopoietic necrosis virus (IHNV).

Gene transfer of a functional antimicrobial peptide through intra-muscular introduction of plasmid DNA to improve immunity against bacterial infections has been accomplished in grouper, *Epinephelus coioides* (Lee et al., 2013). Epinecidin-1 plasmid DNA construct was electrotransferred into the muscle of grouper and its effect on subsequent infection with *Vibrio*

vulnificus was examined. Fish expressing the plasmid DNA had significantly lower bacterial counts and immune-related genes were up-regulated compared to controls. Moreover, AMPs directly injected into non-model fish species conferred their protection against bacterial pathogens. Pleurocidin amide, a peptide derived from flounder (*Pleuronectes americanus*) along with a cecropin-melittin hybrid peptide (CEME) were proven to be potent antimicrobial agents with *in vivo* activity against the pathogen *V. anguillarum* in coho salmon (Jia et al., 2000). Fish that received either pleurocidin amide or CEME survived longer following *V. anguillarum* challenge and had significantly lower mortalities than controls. Similarly, Pan et al. (2007) demonstrated that epinecidin-1 peptide treatment promoted a significant increase in the survival of tilapia (*Oreochromis mossambicus*) and grouper (*E. coioides*) after *Vibrio vulnificus* infection.

Antimicrobial peptides from Ictalurid Species

Catfish is one of the best studied model species for teleost immunity. Relevant immune-related genes for innate immunity have been characterized in catfish, including pattern recognition receptors, antimicrobial peptides, complements, lectins and cytokines (Gao et al., 2012; Pridgeon et al., 2012; Zhang et al., 2012). Two cysteine-rich AMP, hepcidin and liver-expressed antimicrobial peptide 2 (LEAP-2), were sequenced and characterized from both channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*) (Bao et al., 2005; Bao et al., 2006). The expressions of these two AMPs were induced in a tissue-specific manner following infection with *Edwardsiella ictaluri* (Bao et al., 2005; Bao et al., 2006). Hepcidin expression was also detected during embryonic and larval development, possibly indicating that it is a self-generated AMP, thereby protecting embryos from bacterial invasion (Douglas et al., 2003). In addition, hepcidin was significantly upregulated in channel catfish in response to *E. ictaluri*

infection as compared to other AMPs, suggesting the important role of hepcidin in defense against the pathogen (Pridgeon et al., 2012).

Moreover, Wang et al. (2006) identified and characterized three distinct NK-lysin AMPs from catfish, but their biological function has not yet been elucidated. Two other AMPs have been characterized from catfish, bactericidal permeability-increasing protein (BPI) (Xu et al., 2005) and parasin 1 (Cho et al., 2002). BPI has an important role in defending against Gram-negative bacteria in the innate immune system and after challenge with *E. ictaluri*, BPI expression was significantly upregulated in channel catfish (Xu et al., 2005).

Cathelicidin: an effective antimicrobial peptide

Cathelicidins, a class of antimicrobial peptides known to have direct and rapid antimicrobial activities, were first identified in bovine bone marrow-derived myeloid cells (Zanetti et al., 1995; Bals and Wilson, 2003; Tomasinsig and Zanetti, 2005). Since then, many cathelicidin-like AMPs have been identified and characterized from a wide variety of organisms including mammals (Kosciuczuk et al., 2012), reptiles (Wang et al., 2008; Wei et al., 2015; Barksdale et al., 2017) and some species of fish (Uzzell et al., 2003; Chang et al., 2006). Apart from other AMPs, cathelicidins share little sequence similarity between the mature peptides but rather, their structure consists of a homologous *N*-terminal region of the precursor peptide, called a cathelin domain, found just after a conserved signal domain (Tomasinsig and Zanetti, 2005). While the C-terminal antimicrobial domain encodes the mature peptide and is highly diverse among species and different peptides (Zanetti et al., 1995). As prepropeptides or inactive precursors, cathelicidins are stored in neutrophil granules. But when required, the active, mature peptide is released upon proteolytic cleavage by elastase and possibly other enzymes in

conjunction with neutrophil degranulation in order to liberate the antimicrobial peptide at sites of infection (Tomasinsig and Zanetti, 2005; Treffers et al., 2005).

Cathelicidins from Fish

Fish cathelicidins are subdivided into two classes—the linear peptides, and those that exhibit a characteristic disulphide bond (Masso-Silva and Diamond, 2014). Several cathelicidins were identified in different fish species but there is limited information on their properties and functions. To date, no cathelicidin was inserted into the genome of another organism to confer immunity to the host against invading pathogens. But the *in vitro* antibacterial activities of fish cathelicidins seemed to demonstrate variability depending on the species (Masso-Silva and Diamond, 2014). For instance, hagfish (*Myxine glutinosa*) cathelicidin, the first identified cathelicidin in fish, was found to inhibit the growth of both Gram-positive and Gram-negative bacteria, but inactive against the fungus *Candida albicans* (Uzzell et al., 2003). In contrast, the cathelicidin found in cod (*Gadus morhua*) was active against Gram-negative bacterial species examined as well as in *C. albicans*, but almost inactive against the Gram-positive species (Broekman et al., 2011). While both rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*) have two cathelicidin genes each (Chang et al., 2006), their *in vitro* antimicrobial activities are different. Rainbow trout cathelicidins are active against *Yersinia ruckeri*, while Atlantic salmon cathelicidins are not (Bridle et al., 2011). The variability in mature peptide sequences of each cathelicidin appear to mediate the antimicrobial activities and is probably a result of a divergent evolution to address specific pathogens (Masso-Silva and Diamond, 2014).

Alligator Cathelicidins

Recently identified cathelicidins from alligator have gained attention due to their potent, broad-spectrum and rapid antimicrobial activities against both Gram-positive and Gram-negative

bacteria, including multi-drug resistant (MDR) bacterial strains (Barksdale et al., 2017; Chen et al., 2017). Previously, the plasma and leukocyte extracts of American alligator (*Alligator mississippiensis*) and other crocodilians have been shown to exhibit antimicrobial activity (Merchant et al., 2003; Merchant et al., 2006; Darville et al., 2010), but no cationic antimicrobial peptides (CAMPs) were identified from their blood or tissues due to the limitations of conventional proteomics tools (Bishop et al., 2015). However, a new bioprospecting particle-assisted proteomics approach to identify CAMPs based on their fundamental and conserved physico-chemical properties was developed in 2015 using advanced mass spectrometry techniques and *de novo* peptide sequencing (Bishop et al., 2015). This technique paved the way for novel antimicrobial peptides including cathelicidins, to be identified and characterized in alligators.

Crocodilians were thought to possess a powerful immune system as they are constantly engaged in territorial fights resulting in injuries. But they seem to recover quickly from open wounds in water, and their serum has been shown to have antibacterial activity greater than that of human serum (Merchant et al., 2003; Merchant et al., 2006). Some of these bacterial infection-defeating compounds found in the blood of alligator (*A. mississippiensis*) are cathelicidin AMPs (Bishop et al., 2015; Barksdale et al., 2017). In fact, the peptide from alligator is being developed as a therapeutic to protect soldiers from wound infections and potential exposure to biothreat agents (George Mason University, 2015).

To date, novel cathelicidins with direct antimicrobial activities were identified and characterized from two alligator species, American alligator (*A. mississippiensis*) (Barksdale et al., 2017) and from the endangered Chinese alligator (*A. sinensis*) (Chen et al., 2017). Tankrathok et al. (2019) recently identified the first cathelicidin from another crocodilian,

Siamese crocodile (*Crocodylus siamensis*) (Cs-CATH), which was also found to possess direct antimicrobial activity against Gram-positive and Gram-negative bacteria but with low cytotoxicity towards mammalian cell lines. Cathelicidin from American alligator (AM-CATH36) was highly active against multiple Gram-negative bacteria, including clinical isolates of MDR *Acinetobacter baumannii* and carbapenem-resistant *Klebsiella pneumoniae* (Barksdale et al., 2017). The peptide was not hemolytic at 300 µg/mL against sheep blood cells and not cytotoxic against A549 human lung epithelial cells after 24 h exposure. Similarly, six cathelicidins (As-CATH1~6) from Chinese alligator have potent antimicrobial activities, but exhibit no peptide sequence similarity with any of the known cathelicidins (Chen et al., 2017). This feature is unique among cathelicidin peptides that almost no homology in the sequence of active AMP was found between class or orders (van Hoek, 2016). Among the six cathelicidins from Chinese alligator, three cathelicidins (As-CATH4~6) were found to be effective not only against 36 strains of bacteria tested, but also against 8 strains of fungi and 7 strains of aquatic pathogens such as *Aeromonas sobria*, *A. hydrophila*, *A. veronii*, *Vibrio harveyi*, *V. parahaemolyticus*, *V. anguillarum*, and *V. cholerae* (Chen et al., 2017). Furthermore, in an *in vivo* mouse model, As-CATH4~6 conferred protection to peptide-injected mice against abdominal bacterial infections through enhanced immune cell recruitment (Chen et al., 2017). In a follow-up study, Guo et al. (2017) found that when As-CATH4 and As-CATH5 (50 µg/g As-CATH4, 10 µg/g As-CATH5) were injected into Chinese mitten crab *Eriocheir sinensis*, immune parameters such as lysozyme, acid phosphatase and alkaline phosphatase activities were enhanced. This indicated that the immune system of crabs could be activated by these peptides and that it could be utilized as novel peptide immunostimulants in crab aquaculture (Guo et al., 2017).

Cathelicidin mechanisms of action against pathogens

The primary mode of action of cathelicidins, like that of other AMPs, is believed to be bacterial membrane disintegration, rather than disruption of bacterial cell wall synthesis, the usual mechanisms observed with antibiotics (Kosciuczuk et al., 2012; Haney et al., 2019). The rapid antimicrobial activity of cathelicidins can be the result of damaged bacterial cell membranes, ultimately leading to lysis and death of bacterial cells (Ramanathan et al., 2002; Hancock and Sahl, 2006; Cole and Nizet, 2016).

Different mechanisms of peptide penetration across bacterial membranes have been proposed. First is the "barrel-stave" model, where the peptides directly integrate into the target membrane in the form of barrel staves, assuming an α-helical structure and creating channels on the membrane (Brogden, 2005). Second, the "toroidal-pore" mechanism describes the interaction of the peptides with bacterial membranes through transient openings with toroidal shape inducing a curvature in the membrane. The peptides remain closely associated with lipid headgroups throughout the process, unlike in the barrel-stave model where peptides are only initiated with the headgroups (Matsuzaki et al., 1996). Third is the "carpet" mechanism where a high concentration of the peptides accumulates on the surface of the cell and dissolves the cell membrane in a detergent-like manner (Pietiainen et al., 2009).

Cathelicidins possess two characteristics as part of their bactericidal mechanism, amphipathic molecular structure and a net positive charge. These structures can allow them to easily bind to the negatively charged bacterial membrane, alter the membrane's orientation and insert into the bilayer membrane. This can induce bacterial membrane permeabilization and cell death (Mansour et al., 2014). Additionally, the presence of a positively-charged peptide residue contributes to its antimicrobial activity since the peptide would have a selective affinity for

anionic prokaryotic membranes. This mechanism decreases peptide transmembrane penetration into mostly neutral eukaryotic membranes (Jiang et al., 2008).

The mechanism for peptide-bacterial membrane interaction may be different between Gram-positive and Gram-negative bacteria. In Gram-positive bacteria, although the thick peptidoglycan surrounding the bacterial cell lacks a negative charge, the cationic peptide can bind to anionic molecules like teichoic and lipoteichoic acids interspersed in the peptidoglycan structure (Scott et al., 1996). This could easily make the transit of cationic AMP like cathelicidins into the cytoplasmic membrane causing perturbations in bacterial membrane (Vollmer and Bertsche, 2008). While Gram-negative bacteria have an outer membrane that acts as a selective barrier and protection, the lipopolysaccharides (LPS) present in abundance on the surface of the outer membrane possess multiple negative charges to which the cationic peptide can bind. The peptides interfere with the lipid packing and leads to increased permeability of the outer membrane, with the peptide migrating to the cytoplasmic membrane exerting its antimicrobial action (Hancock and Diamond, 2000; Reddy et al., 2004).

Interestingly, cathelicidins are less cytotoxic to host cell membranes (Wei et al., 2015; Barksdale et al., 2017). This may be due to cell selectivity of AMPs, i.e. they selectively inhibit microorganisms without being significantly toxic to host cells (Matsuzaki et al., 2009). The molecular basis for this cell selectivity is the cationic property of AMPs which makes it more attracted to the negatively charged membranes of bacterial cells, thereby exerting its antimicrobial activity. The absence of charge attraction between the cationic peptides and the neutral cell surface of mammalian cells supports anticipated safety for future therapeutic use (Duwadi et al., 2018). Teleosts have cell surface structures comparable to those of mammalian

cells (Sunyer, 2013), and previous studies suggest lack of cytolytic activity in normal cells of fish injected with peptides (Jia et al., 2000; Pan et al., 2007).

Other functions of cathelicidins

Additional important functions of cathelicidins involved host immune modulation and disease resistance, including promotion of angiogenesis and wound healing (Wong et al., 2013). Moreover, recent studies confirmed that their effectiveness to kill microbes and immune modulating activities are more potent than other AMPs previously characterized. For instance, Wei et al. (2015) identified and characterized the first cathelicidin from sea snake, *Hydrophis cyanocinctus* and found that it can induce microbial membrane permeabilization resulting in cellular disruption of both Gram-positive and Gram-negative bacteria. Cathelicidins from Chinese alligator were also found to modulate immune responses such as induction of chemokine production and recruitment of immune cells to the site of infection (Chen et al., 2017).

Bacterial resistance to antimicrobial peptides

Bacterial resistance to antibiotics or drugs has been called one of the world's most pressing public health problems. Antibiotic resistance occurs when bacteria change in some way that reduces or eliminates the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infections. Although AMPs are able to inhibit bacterial growth by causing by intervening with microbial metabolism in various ways, cases of resistance to antibiotic peptide classes have also been reported (Maria-Neto et al., 2015; Cole and Nizet, 2016). Bacterial resistance to AMPs can be observed by bacterial mechanisms such as removal of AMPs from their site of action in the bacterial membrane (Otto, 2009).

There are several mechanisms of AMP resistance in bacteria. First, a common strategy used by bacteria in the resistance process is to modify their cell membranes. For instance, bacteria incorporate positively charged molecules into their cell surface in order to reduce the interaction and binding of cationic AMPs (Andersson et al., 2016). Other bacterial mechanisms that reduces the attachment of peptides to bacterial membranes involve modifications in the polysaccharide layer, lipid A alterations and cell wall thickening of some bacterial species (Maria-Neto et al., 2015; Andersson et al., 2016; Cole and Nizet, 2016). Second, some AMPs are inactivated by proteolytic degradation when bacteria release extracellular proteases (Schmidtchen et al., 2002). Aureolysin, a metalloprotease released in large amounts by *Staphylococcus aureus*, was found to degrade cathelicidin LL-37 by cleaving C-terminal peptide bonds (Sieprawska-Lupa et al., 2004). Third, bacterial surface molecules such as proteins and polysaccharides may directly bind AMPs, thereby blocking access of the peptides to the cytoplasmic membrane target of action and the formation of lytic pores (Cole and Nizet, 2016). Fourth, biofilm formation is an important mechanism of bacterial resistance to antimicrobial compounds and environmental stresses (Dufour et al., 2010). The biofilm matrix produces an extracellular polymeric substance (EPS) where the cells are embedded and biofilm-formation bacteria were known to resist most conventional antibiotics, but do not resist several antimicrobial peptides that act as antibiofilm agents (Di Luca et al., 2014; Maria-Neto et al., 2015).

AMP's mode of action results from the disturbance of cell membrane integrity and these peptides can act in different cell targets including DNA, RNA, regulatory enzymes and other proteins (Maria-Neto et al., 2015). AMPs can be a promising alternative to classic antibiotics; however, the development of AMP-resistant strains will be inevitable. Understanding of

bacterial resistance against AMPs is essential to discriminate the pattern of resistant bacterial strains and to propose the appropriate treatment.

Applications of CRISPR/Cas9 in transgenic fish

The demand for fish lines having enhanced phenotypic attributes such as rapid growth, high fertility, disease resistance and stress tolerance present major challenges to aquaculture, one of the fastest-growing sectors in the food industry. In fact, a number of studies focused on investigating the molecular mechanisms of genes associated with traits of interest. Consequently, molecular biology research has addressed sequencing and characterization of non-model fish genomes, which in turn has led to understanding their genomic functions more precisely. The genetic modification or reverse genetics approach has been utilized for better understanding of gene functions with regards to phenotypic effects (Barman et al., 2017). Traditional gene modification methods were sometimes unsuccessful due to their inefficient, time-consuming and labor-intensive processes (Gaj et al., 2013). In recent years, new approaches in the field of reverse genetics have emerged that enabled investigators to manipulate virtually any gene in a diverse range of cell types and organisms. Gene editing is one of the approaches in reverse genetics where engineered nucleases are utilized to create site-specific double-strand breaks (DSBs) at specific genomic locations to enable a broad range of genetic manipulations (Gaj et al., 2013).

The most popular and widely used gene editing technology is the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology which was originally derived from bacterial adaptive immune system (Jinek et al., 2012). The system consists of a guide RNA which determines the targeted sequence in the genome and Cas9, an endonuclease. Cas9 will induce DSB when introduced into the cell and depending on

the goal, genes can be mutated, edited or inserted (Doudna and Charpentier, 2014). Compared to other techniques that utilize artificial nucleases such as zinc finger nucleases (ZFN) and transcriptional activator-like effector nucleases (TALEN), CRISPR/Cas9 system is preferred by most laboratories because of its simplicity, efficiency, accuracy, lower cost, lower toxicity and higher germline transfer of mutations (Varshney et al., 2015).

Tremendous applications of CRISPR/Cas9 system in model fish species such as medaka and zebrafish were seen in the past few years (Jao et al., 2013; Ansai and Kinoshita, 2014; Gonzales and Yeh, 2014; Hisano et al., 2015). Moreover, CRISPR/Cas9 technology was successfully utilized in several non-model fish species including tilapia (Li et al., 2014), Atlantic salmon (Edvardsen et al., 2014), common carp (Zhong et al., 2016), rainbow trout (Cleveland et al., 2018), rohu (Chakrapani et al., 2016) and channel catfish (Elaswad et al., 2018; Khalil et al., 2017). The applications of gene editing technologies are more relevant in non-model species especially in farmed aquaculture fish because of the economically important traits that would be improved, producing a superior fish within a few generations.

CRISPR/Cas9 knock-out system in fish

Current CRISPR/Cas9-based gene editing in fish can easily generate knock-out models by disrupting the gene sequence, but its efficiency for creating models that require insertion of foreign genes (also known as gene knock-ins) into endogenous genomic loci has been very poor and/or limited (Kimura et al., 2014; Yang et al., 2014). When an induced DSB is introduced into the DNA, the cell can repair the damage either by non-homologous end-joining (NHEJ) where there is no donor DNA, or homology directed repair (HDR) which requires a donor DNA (Gaj et al., 2013; Ansai and Kinoshita, 2014). The DSB repair process by non-homologous end-joining (NHEJ) is error prone, and it can result in loss or gain of some nucleotides i.e. insertion,

deletions, or substitutions which is favored for genetic knock-outs. The efficiency of NHEJ-mediated mutation is high because it occurs throughout the cell cycle and is a dominant repair system in vertebrates (Moore and Haber, 1996).

CRISPR/Cas 9 knock-out models in fish are more vastly explored than knock-in models. In model fish such as medaka, one of the pioneer studies utilizing CRISPR/Cas9 successfully obtained somatic mutation at a protein deglycase (DJ-I) locus with 87 % efficiency (Ansai and Kinoshita, 2014). CRISPR/Cas9-mediated knock-out zebrafish models are also popular and have been used to model several diseases (Liu et al., 2018; Ramanagoudr-Bhojappa et al., 2018). In non-model fish, many studies have been successful in creating loss of gene function mutations that produced desirable phenotypes in farmed fish (Khalil et al. 2017; Kishimoto et al. 2018; Li et al. 2014; Zhong et al. 2016). For instance, Khalil et al. (2017) and Kishimoto et al. (2018) achieved high rates of mutagenesis in the target protein-encoding sites of myostatin, a negative regulator of skeletal muscle mass, in channel catfish (*Ictalurus punctatus*) and red sea bream (*Pagrus major*), respectively. Knock-out of myostatin gene resulted to 30 % increase in mean body weight of gene-edited channel catfish fry and a 16 % increase in skeletal muscle or the edible muscle parts of a market size red sea bream. Similarly, Zhong et al. (2016) successfully disrupted the muscle suppressor gene *mstn* in common carp (*Cyprinus carpio*), and resulting in mutated fish that displayed significantly enhanced muscular growth, while other mutated genes such as *sp7*, *runx2* and *spp1* showed severe bone defects.

CRISPR/Cas 9 was also utilized to generate germ cell-free salmon to avoid introgression of farmed salmon into wild populations (Wargelius et al., 2016). The authors knocked out dead end (*dnd*) gene, a factor essential for germ-cell survival and generated germ cell-less salmon, which also lacked pigmentation through induced mutations for the *alb* gene. Edvardsen et al.

(2014) also knocked out two genes, tyrosinase and solute carrier family 45, in Atlantic salmon to better understand pigmentation, and mutated fish exhibited complete lack of pigmentation. In Nile tilapia (*Oreochromis niloticus*), CRISPR/Cas9 was used to disrupt selected genes involved for sex determination such as *nanos2*, *nanos3*, *dmrt1*, and *foxl2*, with efficiencies as high as 95% (Li et al., 2014). Subsequently, investigators found germ-cell deficient gonads and masculinization in Nile tilapia.

In channel catfish, two immune-related genes correlated with disease progression, the toll-interleukin 1 receptor domain-containing adapter molecule (TICAM 1) and the rhamnose-binding lectin (RBL), were knocked out using CRISPR/Cas9 and achieved mutation rates as high as 93 % (Elaswad et al., 2018). But further work is needed to know the effects of TICAM 1 and RBL gene knockout on the immune response and disease resistance of channel catfish.

CRISPR/Cas9 knock-in system in fish

Knock-in of exogenous DNA fragments can be performed through a different DNA repair pathway: HDR. This pathway requires a homologous DNA template to promote DNA repair through homologous recombination (HR), allowing the integration of any desirable DNA sequence at the target site (Cornet et al., 2018).

However, HDR is still a challenging approach due to the low rate of DSB repair by HR compared to NHEJ (Maruyama et al., 2015). HR occurs only during the late S and G2 phase and not throughout the cell cycle. Therefore, the reported efficiency of homology-directed integration was ranged between 1-10 % (Maruyama et al., 2015; Moreno-Mateos et al., 2017). Efforts were geared towards enhancing HDR, including control of the cell cycle events and regulation of expression of key repair pathway proteins (Srivastava and Raghavan, 2015), but such manipulations may be undesirable as they can alter the cellular response to DNA damage at

other non-target sites in the genome (Song and Stieger, 2017). Thus far, successful integration of exogenous genes can be achieved by designing optimal DNA donor templates, such as a plasmid donor with at least 1–2 kb of total homology is usually used for creating large sequence changes in the presence of target cleavage (Dickinson et al., 2013; Yang et al., 2013) and for small sequence changes, single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) sequences are used (Song and Stieger, 2017). Researchers have also attempted to enhance the more precise HDR pathway and/or suppress the error prone NHEJ pathway by targeting key factors such as manipulating the cell cycle phase, stimulating HDR-promoting factors, such as the RAD51 recombinase, or inhibiting NHEJ factors by small molecules or shRNA-mediated silencing (Pinder et al., 2015; Mateo-Gomez et al., 2017; Schimmel et al., 2017; Zelensky et al., 2017).

Designing an optimal donor DNA template can contribute to increased HDR frequencies (Song and Stieger, 2017), which is favorable for genetic knock-in studies. For instance, Hisano et al. (2015) modified the donor plasmid by using short homologous sequences (20–40 bp) flanked by two sgRNA target sequences (also known as double cut donors), and observed precise integration rate ranged between 60-77 % of an exogenous *mCherry* or *eGFP* gene into the targeted genes (*tyrosinase* and *krtt1c19e*) in zebrafish. Conversely, some studies have shown that the efficiency of recombinatorial repair increases as the length of homology arms increases (Li et al., 2014, Song and Stieger, 2017; Zhang et al., 2017). Zhang et al. (2017) observed that increasing the length of homology arms from 50 bp to 300 bp led to increased HDR efficiency in 293 T cells, with a 77% precise insertion rate observed in homology arms of 300 bp as compared to 63% insertion rate for 50 bp homology arms. Some studies used longer homology arms of 1 kb or more and achieved 12-58 % HDR rate (Byrne et al., 2015; Song and Stieger, 2017), but

shorter homology arms are more accessible for insertion in the target site during the simultaneous cleavage of genomic and plasmid DNA (Zhang et al., 2017).

Targeted gene disruption *via* HR was recently accomplished using CRISPR/Cas9 in the large-bodied teleost, rohu carp (*Labeo rohita*) (Chakrapani et al., 2016). The investigators successfully disrupted rohu Toll-like receptor 22 (TLR22) gene involved in innate immunity and generated null mutants that lacked TLR22 mRNA expression. However, a targeted gene insertion *via* HR utilizing CRISPR/Cas9 in a farmed fish has not yet been reported.

Targeting the non-coding sequences using CRISPR/Cas9 system

Eukaryotic genomes comprise of protein coding genes and a bulk of noncoding DNA (Amaral et al., 2008). Scientists once thought that noncoding DNA was “junk,” with no known purpose. However, recent studies have indicated that they are integral to the function of cells, particularly the control of gene activity (Maumus and Quesneville, 2014; Plank and Dean, 2014). For instance, it is now known that channel catfish genome has 100 % function (Liu et al., 2016). Every base has a function, and noncoding sequences act as regulatory elements and as structural elements such as repetitive DNA sequences (Yuan et al., 2018). Other regions of noncoding DNA also provide instructions for certain kinds of RNA molecules such as transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) (Plank and Dean, 2014; Liu et al., 2016).

Protein coding sequences become the target of many CRISPR/Cas9 applications, mostly in generating small insertion and deletion (indel) mutations to disrupt the open reading frames of protein coding genes (Cong et al., 2013; Li et al., 2014). On the contrary, mutation of non-coding sequences using CRISPR/Cas9 system is often difficult to achieve because small indels caused by a single mutation do not result in loss of function (Li et al., 2019). Targeting the non-

coding sequences either through loss-of-function or gain-of-function approaches can be advantageous since these regions are proposed to affect the expression of neighboring or distant genes by acting as signaling, guiding, sequestering or scaffolding molecules (Canver et al., 2017; Goudarzi et al., 2019). This could also be a disadvantage as it might affect multiple genes resulting in off-target effects. Some studies have successfully obtained large genomic deletion using dual guide RNAs (gRNAs) in mammalian cells and animal models such as mouse and zebrafish (Zhao et al., 2017; Xiong et al., 2018). Deletion mutations of up to 900 bp were recently achieved in channel catfish targeting toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) using a single guide RNA (Elaswad et al., 2018). Moreover, Li et al. (2019) reported that CRISPR/Cas9 system could effectively generate desirable non-coding sequences mutants in Nile tilapia (*Oreochromis niloticus*). The researchers used one or dual gRNA guided Cas9 nuclease and short ssDNA as a donor to achieve specific deletion of noncoding sequences including microRNA and 3' untranslated region (UTR) in tilapia, an important fish for studying sex determination and evolution. These findings have paved the way for gene editing in aquaculture fish, aiming towards all-inclusive improvements of production and/or performance traits.

Mosaicism in transgenic fish

Genetic mosaicism occurs when there is more than one genotype in an individual developed from a single fertilized egg. It can result from natural mechanisms such as chromosome non-disjunction, anaphase lag, endoreplication, and mutations arising during development (Taylor et al., 2014). Alternatively, it can result from laboratory manipulation *via* transgenesis or genome editing.

In the conventional non-targeted gene transfer, delayed transgene integration during embryonic cell division could result in a mosaic fish (Dunham and Winn, 2014). Also, in non-targeted gene insertion, insertion site cannot be controlled which could create random DSBs, thus repair process would be inefficient (Lombardo et al., 2011). This may also result in undesired mosaicism. When the transgene is integrated in only one cell group or tissue but not into germ cells, gene transmission to the offspring would be difficult (Maclean, 1998; Dunham and Winn, 2014).

With the CRISPR/Cas9 system, programmable nuclease-induced DSB generation at the desired gene insertion site using sgRNA and Cas9 could enhance the efficiency of gene insertion through homology-directed repair (HDR) and could lead to reduced mosaicism (Mehravar et al., 2019). But still, mosaicism is inevitable even with CRISPR/Cas9 technology. Mechanisms of mosaicism resulting from CRISPR/Cas9 gene editing system includes Cas9 translational delay, random DSBs and repair process, properties of the target locus, and concentration and multiplicity of CRISPR/Cas9 components (Niu et al., 2015; Sato et al., 2015; Tu et al., 2015; Raveux, et al., 2017). Several possible strategies have been proposed to efficiently reduce or eliminate mosaicism in CRISPR/Cas9 system, but it still depends on the purpose of the study and type of species used. The timing of Cas9 expression relative to DNA replication in the zygote can overcome the problem of mosaicism (Sung et al., 2014). Injecting the CRISPR/Cas9 components into very early-stage zygotes can increase the efficiency of the genome editing process, with the divided cells carrying the exogenous donor DNA during replication. Early delivery of CRISPR components have reduced mosaicism in mice (Hashimoto et al., 2016), sheep (Vilarino et al., 2017) and cattle (Lamas-Toranzo et al., 2019). Another strategy is to use long ssDNAs as repair donors in combination with ctRNP (crRNA, tracrRNA and Cas9 protein)

complexes (Quadros et al., 2017). This could lead to precise genome editing by CRISPR/Cas9 system. Germline modification can be another strategy to limit mosaicism and could either be direct or indirect (Mehravar et al., 2019). Direct germ line modification uses gene-edited spermatogonial stem cells (SSCs) as donors for transplantation into testis. This technique has produced non-mosaic mutant rats and mouse strains (Chapman et al., 2015; Wu et al., 2015). For indirect germline modification, genetically modified somatic cells are used as nuclear donors for somatic cell nuclear transfer (SCNT) into enucleated germ cells, and resulted to reduced mosaicism in pigs and goats (Ni et al., 2014).

Off-target effects in CRISPR/Cas9 system

CRISPR/Cas9 technology has proven to be an efficient and versatile tool in a wide variety of genome editing applications. However, the off-target effects, mainly due to the unintended cleavage of DNA at sites whose sequences show mismatches with the guide RNA (gRNA) (Wang and Wang, 2019), are a major concern in CRISPR/Cas9 system. Nonspecific and unintended alterations to the genome may result from off-target editing and can often lead to inadvertent effects on the organism (Gratacap et al., 2019). Off-target effects are mainly divided into the following types: (1) in many instances, non-recognition of Cas9/gRNA of more than three mismatched DNA sites; (2) Cas9/gRNA does not recognize and edit DNA sites with any number of mismatches (within 10–12 bp) near the protospacer adjacent motif (PAM); (3) the higher the Cas9/gRNA concentration, the greater the possibility of off-target effects (Hsu et al., 2013; Pattanayak et al., 2013).

Various strategies to minimize off-target effects have been reported (Kim et al., 2015; Zhang et al., 2015; Chen, 2019). First, the gRNA can be modified to create a truncated version of less than 20 nucleotides to weaken gRNA-DNA duplex stabilities at the off-target sites. This

strategy improved target specificity and decreased off-target cleavage by 5000-fold in some studies (Pattanayak et al., 2013; Fu et al., 2014). Second, the concentration of Cas9-gRNA complex should be controlled by titrating the amount of Cas9 and gRNA delivered into the cells (Zhang et al., 2015). Optimizing the concentration of the complex may improve the specificity of Cas9 to cleave DNA at the target cut site (Hsu et al., 2013). Cas9-gRNA complex cleaves chromosomal DNA almost immediately after introduction to cells and also degrades rapidly, so direct delivery of purified Cas9 protein and gRNA is important and can reduce off-target effects (Kim et al., 2014; Zhang et al., 2015). Third, to strengthen the on-target stability of the CRISPR/Cas9 system, Cas9 nickase mutant or dimeric Cas9 protein complexed with pairs of gRNAs can be used (Ran et al., 2013). This strategy can initiate double checkpoints for target recognition by increasing the number of mutated base pairs at the target site, thereby reducing off-target frequency (Ran et al., 2013; Frock et al., 2015). However, these strategies require complex components of the CRISPR/Cas9 system and could present challenges in gene delivery (Chen, 2019). It should also be carefully considered that off-target effects might be cell-type-specific and dependent on the integrity of double-stranded breaks (DSBs) repair pathways of a particular cell type (Duan et al., 2014).

Future perspectives for improving disease resistance in fish

Infectious disease is one of the primary constraints to aquaculture production, thus, effective approaches in controlling infectious diseases are highly desirable. Genome editing together with transgenesis can allow for precision breeding, enhancing important traits in aquaculture including growth and reproductive performance, tolerance to stressors and disease resistance. Effective peptides with broad spectrum antimicrobial activity should be able to be inserted into the genome of a farmed fish using a variety of genetic engineering techniques to

produce a disease-resistant fish. A transgenic fish encoding an antimicrobial peptide can confer immunity in fish since it will be protected by the expressed peptide from early in development. Also, an innately disease-resistant fish would not require specific vaccination for certain pathogens and thus will provide an economical solution to bacterial disease problems (Dunham et al., 2002).

With the identification of several disease-susceptibility genes, targeted gene manipulation can now become almost limitless. To date, most CRISPR/Cas9 gene editing in fish focused on genetic knockouts. However, developments in targeted gene insertion to introduce disease resistance genes can be expected in the near future. Immunity and disease resistance in Rohu carp has been explored *via* CRISPR/Cas9 gene editing where it was focused on targeted disruption of TLR22 gene involved in innate immunity (Chakrapani et al. 2016). This area of research can help improve our understanding of host responses to fish infection and can serve as guide to effective treatment regimes. CRISPR/Cas9 technology also holds great promise to advance our understanding and modification of gene function using improved fish cell lines. For instance, modified fish cell lines can be used to efficiently produce viruses for future vaccine development by knocking out key components of the interferon pathway (Dehler et al., 2016).

In the current studies, the primary goal was to produce a disease resistant fish in a shorter time frame compared with traditional selection. However, there are ethical concerns and environmental risks associated with gene-edited organisms. Approval for food-marketed transgenic animals requires a lengthy process. Growth hormone modified transgenic salmon were approved for human consumption by the FDA and the Canadian Food Inspection Agency after 20 years in regulatory limbo (Waltz, 2016). The regulation for gene-edited fish should logically be less stringent since modification was done at precise chromosomal location,

producing minimal off-target effects (Barman et al., 2017). The researcher must carefully assess the potential risks regarding how the technology affects the individual genome in addition to its environmental impacts. To ensure continuous genetic improvement, researchers must carry out consistent integration of gene editing technologies into breeding programs. It is also necessary to cautiously determine any potential unintended pleiotropic effects before commercial application.

Objectives

1. Evaluate the ability of four antimicrobial peptides: moth cecropin, alligator cathelicidin, sea snake cathelicidin and flounder pleurocidin to inhibit catfish pathogens *in vitro*.
2. Assess the efficiency of four antimicrobial peptides: moth cecropin, alligator cathelicidin, sea snake cathelicidin and flounder pleurocidin to protect channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) against an infection caused by the pathogen *Aeromonas hydrophila*.
3. Determine the effectiveness of cathelicidins in protecting channel catfish and hybrid catfish from *Edwardsiella ictaluri* infection. The immune stimulation effect of cathelicidins from alligator and sea snake will be compared to other AMPs such as cecropin and pleurocidin.
4. Assess the efficiency of different donor DNA templates: namely double-stranded DNA sequences carrying two different promoters and plasmid DNA donors on the integration rate, embryo mortality, embryo hatchability and early fry survival. Transgene expression, changes in body weight and serum bactericidal activity were also determined.

5. Compare the gene integration efficiency of the different transgene delivery methods namely CRISPR/Cas9 microinjection method and CRISPR/Cas9 electroporation in delivering cathelicidin gene in channel catfish.

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CHAPTER TWO

Evaluation of Antimicrobial Peptides for Effectiveness Against Catfish Pathogens

Abstract

One of the major goals in aquaculture is to protect fish against infectious diseases as disease outbreaks could lead to economic losses if not controlled. Antimicrobial peptides (AMPs), a class of highly conserved peptides known to possess direct antimicrobial activities against invading pathogens, were evaluated for their ability to protect channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) against infection caused by the fish pathogen *Aeromonas hydrophila* ML09-119. To identify effective peptides, the minimum inhibitory concentrations (MICs) against pathogens namely *Edwardsiella ictaluri* S97-773, *E. piscicida* E22-10, *A. hydrophila* ML09-119, *A. veronii* 03X03876 and *Flavobacterium columnare* GL-001 were determined *in vitro*. Cathelicidins derived from alligator and sea snake exhibited potent and rapid antimicrobial activities against the tested catfish pathogens as compared to cecropin and pleurocidin AMPs, and ampicillin, the antibiotic control. When the peptides (50 µg/mL) were injected into fish and simultaneously challenged with *A. hydrophila* through immersion, increased survival rates in channel catfish and hybrid catfish were observed in both cathelicidin (alligator and sea snake) treatments as compared to other peptides and the infected control. Bacterial numbers in the kidney and liver of channel catfish and hybrid catfish also decreased significantly after 24- and 48-h post challenge infection. These results show the potential of cathelicidin to protect catfish against bacterial infections and suggests an approach that overexpressing the peptide in transgenic fish may provide a method of decreasing bacterial disease problems in catfish.

1. Introduction

One of the key components of the innate immune system present in virtually all species are the antimicrobial peptides (AMPs). They are also known as host defense peptides (HDPs) to encompass a myriad of biological processes that are influenced by these biomolecules including antimicrobial activity, immune modulation, anti-inflammatory and antibiofilm activity (Nijnik and Hancock, 2009; Haney et al., 2019). AMPs are effective against a wide variety of microorganisms including both Gram-positive and Gram-negative bacteria, fungi and some viruses (Hancock and Sahl, 2006). Their small, cationic characteristics allow them to easily diffuse in microbial membranes, thereby exerting rapid antimicrobial action but have low toxicity to animal cells. Electrostatic interaction between the positively charged peptide and the negatively charged bacterial membrane allows peptide binding to bacterial membranes (Hancock and Lehrer, 1998). Pathogens will less likely develop resistance to AMPs since these peptides have several modes of action and majority of them do not target metabolic pathways (Andrès and Dimarcq, 2004). Well-known examples of cationic antimicrobial peptides with broad-spectrum antimicrobial activity *in vitro* and *in vivo* are the cecropins, defensins, lactoferrins, hepcidins, melittins, pleurocidins and cathelicidins (Yamauchi et al., 1993; Hancock and Lehrer, 1998; Hsieh et al., 2010; Pan et al., 2011).

Expression of genes encoding peptides with *in vitro* antimicrobial activity can result in enhanced resistance to bacterial pathogens in transgenic fish. Cecropins are one of the best-studied antimicrobial peptides in fish, and *in vitro* studies have shown that it has positive bactericidal activity to fish pathogens such as *Pseudomonas flourescens*, *Aeromonas hydrophila* and *Vibrio anguillarum* (Sarmasik and Chen, 2003; Chiou et al., 2006). When cecropin B gene driven by cytomegalovirus promoter was inserted into channel catfish, improved disease

resistance and survival of F1 generation was observed when challenged with the pathogens *Flavobacterium columnare* and *Edwardsiella ictaluri* (Dunham et al., 2002). Similarly, Chiou et al. (2014) demonstrated that homozygous F2 and F3 transgenic rainbow trout expressing cecropin B exhibited resistance to infection by *Aeromonas salmonicida* and infectious hematopoietic necrosis virus (IHNV). Moreover, pleurocidin amide, a peptide derived from flounder (*Pleuronectes americanus*) along with a cecropin-melittin hybrid peptide (CEME) were proven to be potent antimicrobial agents with *in vivo* activity against the pathogen *V. anguillarum* in coho salmon (Jia et al., 2002).

Recently, cathelicidins derived from American alligator (*Alligator mississippiensis*) were shown to have a strong activity against some Gram-negative bacteria as well as in multi-drug resistant bacterial pathogens such as *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Barksdale et al., 2016; Barksdale et al., 2017). Two novel cathelicidins (As-CATH4 and 5), derived from Chinese alligator (*A. sinensis*), were shown to have strong antimicrobial activity against some aquatic bacteria and enhanced the immune-protective efficiency of Chinese mitten crab (*Eriocheir sinensis*) following *A. hydrophila* challenge (Guo et al., 2017). Currently, there are limited studies on the applications of cathelicidin from alligator and none in fish, but three cathelicidin analogs derived from other vertebrates are now undergoing clinical trials as novel anti-infective drugs. These are cattle indolicidin analogue omiganan (MBI-226, CPI226), cattle indolicidin analogue MX-594AN, and pig protegrin-1 analogue iseganan (IB-367) (Isaacson, 2003; Steinstraesser et al., 2011; Elad et al., 2012). It is believed that the antimicrobial effect of cathelicidins is far more rapid than traditional antibiotics (Zanetti, 2004; Wang et al., 2008). Their mode of action is to target bacterial cell membranes, damaging and puncturing the membrane resulting to cell death (Kosciuczuk et al., 2012).

Catfish is the leading aquaculture industry in the United States, but is faced with setbacks especially disease outbreaks. Among these have been severe outbreaks of enteric septicemia of catfish (ESC) caused by *Edwarsiella ictaluri*, motile *Aeromonas* septicemia (MAS) caused by *A. hydrophila*, columnaris disease caused by *Flavobacterium columnare*, and an emergent catfish septicemia caused by *E. piscicida* (Griffin et al., 2018; MSU, 2012). Infectious diseases affecting the catfish industry have caused producers millions of dollars of direct fish losses each year. To reduce disease incidences and decrease economic losses, integration of effective approaches to control these diseases are highly desirable. Screening of antimicrobial peptides for their functionality and effectiveness to inhibit these aquatic pathogens would be advantageous since it may provide an alternative approach to control or reduce disease severity aside from conventional vaccination and use of antibiotics. Further, if an effective peptide is identified, it can be inserted into the catfish genome using a variety of genetic engineering techniques to produce a disease-resistant fish. A transgenic fish encoding an antimicrobial peptide can confer immunity in fish since it will be protected by the expressed peptide from early in development. Also, an innately disease-resistant fish would not require specific vaccination for certain pathogens and thus will provide an economical solution to bacterial disease problems (Dunham et al., 2002). Therefore, the ability of four antimicrobial peptides, cecropin, cathelicidin from alligator, cathelicidin from sea snake and pleurocidin was investigated to inhibit the growth catfish pathogens *in vitro*. Also, an *in vivo* challenge test was conducted to assess the efficiency of the peptides to protect channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) against an infection caused by the pathogen *A. hydrophila*.

2. Materials and Methods

2.1. Experimental fish and conditions

Channel catfish and hybrid catfish (mean ± SD weight 8.40 ± 2.55 g) were obtained from stocks maintained at the Auburn University Fish Genetics Laboratory and acclimated in dechlorinated tap water at 25-28°C for 48 h prior to experiment. Fish were placed in a 365-L tank (height, width and length were 57 cm, 72 cm and 3 m, respectively) for acclimation with continuous aeration and water flow. Throughout the duration of the study, water temperature was 28.5 ± 0.4ºC, pH was 7.0 ± 0.2, dissolved oxygen was 6.60 ± 0.50 mg/L, nitrite was 0.50 ± 0.2 mg/L, alkalinity was 80 mg/L and ammonia concentration was 0.50 ± 0.05 mg/L. Fish handling and treatment procedures used during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC).

2.2. Synthesis of peptides

The peptides used in this study (Table 1) were custom ordered from Biomatik Corporation (Cambridge, Ontario, Canada). The synthetic peptides were purified and analyzed by HPLC and Mass Spectrometry to confirm purity of greater than 95% (Biomatik). Peptides were dissolved in distilled water at 1 mg/ml as stock solution before use.

Table 1 Peptide sequences used in the *in vitro* and *in vivo* tests against catfish pathogens.

Peptide	Sequence	Reference
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	Dunham et al. 2002
Cathelicidin alligator	GLFKKLRRKIKKGFKKIFKRLPPIGVGVSIPLAGKR	Barksdale et al. 2017
Cathelicidin sea snake	KFFKRLLKSVRRAVKKFRKKPRLIGLSTLL	Wei et al. 2015
Pleurocidin amide	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2	Jia et al. 2000

2.3. Bacteriology

Field isolates of four catfish pathogens namely *Edwardsiella ictaluri* (strain S97-773), *Edwardsiella piscicida* (strain E22-10), *Aeromonas hydrophila* (strain ML09-119), *Aeromonas veronii* (strain 03X03876) were provided by the USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL. *Flavobacterium columnare* (strain GL-001) was isolated from a diseased fish following a columnaris outbreak in our laboratory in 2016. All strains were stored as glycerinated stocks at -80°C until they were thawed for use.

For the pathogen challenge test, *A. hydrophila* used in this study was the virulent strain (ML09-119), as confirmed by growth on agar plates containing *myo*-inositol. Virulent *A. hydrophila* (vAh) strains possess a unique metabolic pathway for *myo*-inositol utilization (Hossain et al., 2014). A frozen glycerol stock of vAh was inoculated into 20 mL tryptic soy broth (TSB; BD Difco, Sparks, Maryland) and grown at 28°C in a shaker incubator at 180 rpm for 24 h. Cultures were then expanded into 1 L TSB and grown under the same conditions for 18-20 h until the cell density reached to approximately 1 x 10^9 cells/mL based on an absorbance value read at 600 nm (OD_{600}). Standard plate counts in triplicates were carried out to enumerate the average number of colony forming units per milliliter (CFU/mL) of bacterial cells used to challenge fish.

2.4. *In vitro* assay

The antimicrobial activities of four peptides against five catfish pathogens were determined as minimal inhibitory concentrations (MICs) using the standard two-fold broth microdilution method (Weigand et al., 2008) with modifications. MIC is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism

after overnight incubation. Briefly, bacteria were grown overnight in nutrient broth (BD Difco, Sparks, Maryland) and diluted to give a final inoculum size of 10^6 CFU/ml. Peptides were serially diluted with nutrient broth to concentrations 3.13, 6.25, 12.5, 25, 50, 100, and 200 µg/mL in 96-well microtiter plates at 50 µL/well. Bacterial inoculum was added to each well (50 µL) and incubated at 28°C for 16-20 h. The lowest concentrations of peptides at which no bacterial growth occurred were recorded as MIC values. Ampicillin, at different concentrations similar to the peptide dilutions, was used as positive control.

2.4.1. Bacterial killing kinetic assay

In vitro bacterial killing kinetics of the peptides were evaluated based on the method described by Wei et al. (2015) with minor modifications. *A. hydrophila* was incubated in TSB to exponential phase and diluted to 10^6 CFU/ml with fresh TSB. The peptides were added to the bacterial suspension to a final concentration of 10 x MIC and incubated at 28°C for 0, 30, 60, 90, 120, and 180 min. An aliquot of 50 µL of a bacterial suspension was extracted at each time point and diluted 1000-fold with fresh TSB. Then, 50 µL of the dilutions were plated on tryptic soy agar (TSA) plates and incubated overnight at 28°C. Viable colonies were counted. Ampicillin (10 x MIC) was used as positive control and sterile distilled water as blank control.

2.5. *In vivo* experiments

Fish used for the challenge were anesthetized with 0.01 % tricaine methanesulfonate (MS-222, buffered with 0.02 % sodium bicarbonate, pH 7.35 to 7.45). The peptides were delivered by intraperitoneal injection of a stock at a concentration of 50 µg/mL. Control fish were injected with saline only. Simultaneous with peptide injection, the adipose fin was individually clipped at its base as described by Zhang et.al. (2016). There were four peptide treatment groups: cecropin B, cathelicidin-alligator, cathelicidin-sea snake and pleurocidin

amide. Each treatment group consisted of three replicates with 10 fish per basket (Figure 1). The wire mesh baskets were labelled with codes and were arranged in a randomized block design. Two types of control were used in this study: infected and non-infected. The infected control group was placed in the infected tank with the peptide treatments, while the non-infected control group was in a separate tank and received only TSB without the bacteria. Before the start of the challenge, water was lowered in the tank to 100 L total volume. One liter of vAh bacterial suspension containing approximately $1.0 \pm 0.5 \times 10^9$ cells was added to the treatment/infected tank resulting in a final concentration of ~$1.0 \pm 0.5 \times 10^7$ cells/mL, while 1 L of TSB only was poured into the non-infected tank. Fish were immersed in static condition for 1 h with aeration. At the conclusion of 1 h static challenge, water was restored. The infected control group, which received saline only, were placed together in one tank with the peptide group, while the non-infected control group was in a separate tank and received no bacteria only TSB. Fish were observed every 4 h and dead or moribund fish were either sampled or removed. To confirm the presence of vAh as the causative agent of mortality, dead or moribund fish were sampled to isolate *Aeromonas* in the kidney and liver. Fish were not fed on the day of the challenge but were given food *ad libitum* the next day after the infection and throughout the experiment.

Figure 1 Experimental set-up for *Aeromonas hydrophila* challenge of channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) injected with antimicrobial peptides. Two 365-L tanks were used, an infected tank and a non-infected tank. The four peptide treatments were intraperitoneally injected into the fish and simultaneously challenged with *Aeromonas hydrophila*. The injected control group received saline injection only. The non-injected control group was in the non-infected tank and received tryptic soy broth only. Each treatment had three replicates with 10 fish in each basket.

2.5.1. Bacterial counts in fish kidney and liver

After 24- and 48-h post infection, three fish from each treatment were randomly collected, anesthetized with 0.01% tricaine methanesulfonate (MS-222), buffered with 0.02 % sodium bicarbonate) and had their kidneys and livers removed. The tissues were immediately diluted to 1000-fold with sterile PBS (pH 7.4) and homogenized. Fifty microliters of the tissue dilutions were plated on nutrient agar, and the plates were incubated at 28°C for 18 h. The number of viable bacterial colonies grown on the plates was recorded and expressed as log colony forming units/gram (CFU/g).

2.6. Statistical analysis

MIC values and time-kill kinetic values were means of three independent experiments. Survival curves for channel catfish and hybrid catfish injected with different peptides and control groups were compared using the Kaplan-Meier test (Goel et al., 2010). Comparison of survival curves of all the treatments were performed using the Log Rank test (Bland and Altman, 2004). Fish mortality data were analyzed using one-way ANOVA and Tukey's multiple comparisons test using R software (R Core Team, 2014). *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. *In vitro* activities of four AMPs against catfish pathogens

Catfish pathogens *E. ictaluri*, *E. piscicida, A. hydrophila*, *A. veronii* and *F. columnare* were selected for the *in vitro* test since they were the common causes of infection in most farms and experimental ponds or tanks. Cathelicidin from alligator consistently inhibited most bacterial growth at concentrations as low as 6.25 µg/mL (Table 2). Cathelicidin from sea snake and cecropin B both inhibited the growth of *A. hydrophila* and *A. veronii* at a concentration of 12.5 µg/mL, while pleurocidin amide inhibited both bacteria at 25 µg/mL. Four out of five strains were ampicillin-resistant, with MIC values greater than 200 µg/mL. Thus, ampicillin was not likely to be effective in inhibiting the pathogens, except for *E. piscicida* (see Table 2).

Table 2 Activity of antimicrobial peptides against catfish pathogens.

Microorganisms	**MIC (μg/mL)**				
	Cecropin B	Cathelicidin alligator	Cathelicidin sea snake	Pleurocidin amide	Ampicillin
Edwardsiella ictaluri	>200	25 (6.16 μM)	>200	>200	>200
Edwardsiella piscicida	100 (26.08 μM)	6.25 (1.54 μM)	>200	200 (73.80 μM)	3.13 (8.96 μM)
Aeromonas hydrophila	12.5 (3.26 μM)	6.25 (1.54 μM)	12.5 (3.44 μM)	25 (9.22 μM)	>200
Aeromonas veronii	12.5 (3.26 μM)	6.25 (1.54 μM)	12.5 (3.44 μM)	25 (9.22 μM)	>200
Flavobacterium columnare	100 (26.08 μM)	100 (24.64 μM)	200 (55.12 μM)	>200	>200

MIC, minimal inhibitory concentration. The results represent mean values of three independent experiments.

To examine the rate of bacterial killing of the peptides against *A. hydrophila*, a standard bacterial killing kinetic assay was performed. The CFUs for both cathelicidins, alligator and sea snake, decreased to zero after 120 min and was comparable to the effect of ampicillin (Table 3). Moreover, the CFUs of both cathelicidins remained zero even on an extended incubation time of 180 min. This implied that the antimicrobial property of cathelicidins was bactericidal (able to kill bacteria) rather than bacteriostatic (inhibit growth or reproduction).

Table 3 Killing kinetics of antimicrobial peptides against *Aeromonas hydrophila*.

	Time					
Peptide	**0 min**	**30 min**	**60 min**	**90 min**	**120 min**	**180 min**
			x 10^3 CFU/ml			
Cecropin B	73 ± 7.0	62 ± 14.4	64 ± 4.9	55 ± 6.1	35 ± 6.1	18 ± 5.3
Cath-Alligator	80 ± 8.0	56 ± 11.1	56 ± 14.2	33 ± 7.5	0	0
Cath-Sea snake	70 ± 7.0	53 ± 10.1	56 ± 10.1	39 ± 7.8	0	0
Pleurocidin	77 ± 4.2	51 ± 11.0	58 ± 3.5	36 ± 11.1	34 ± 4.2	20 ± 6.0
Ampicillin	35 ± 7.5	77 ± 10.0	42 ± 11.5	20 ± 6.5	0	0
Control	63 ± 10.4	99 ± 15.0	126 ± 9.2	334 ± 19.0	622 ± 11.0	1124 ± 7.0

A.hydrophila was mixed with different peptides at a concentration of 10 X MIC (minimum inhibitory concentration) for 0, 30, 60, 90, 120 and 180 min. Ampicillin (10 X MIC) was used as positive control. The MICs of cecropin, cathelicidin from alligator (cath-alligator), cathelicidin from sea snake (cath-sea snake) and pleurocidin against *A. hydrophila* were 12.5, 6.25, 12.5 and 25 μg/mL, respectively. The results represent mean values of bacterial colonies counted from three independent experiments ± standard error (SEM).

3.2. Protection against *Aeromonas hydrophila* infection

To further confirm the efficacy of cathelicidins for inhibiting bacterial growth, *in vivo* protection studies against *A. hydrophila* were conducted. Infected channel catfish in the pleurocidin and control group started dying at day 2 of the challenge and had the lowest survival rate at day 8 at challenge termination (Figures 2 and 3). Variable effects were observed in hybrid catfish where the control, pleurocidin and cathelicidin-sea snake groups started dying early on, but both the control and cecropin groups had the lowest survival rate. Increased survival rates in channel catfish and hybrid catfish were observed for both cathelicidins compared to other peptides and the infected control. In channel catfish, high survival rates were observed in fish injected with cathelicidin-sea snake (70 %) and cathelicidin-alligator (67 %) ($P < 0.01$) (Figure 3A). While in hybrid catfish, survival rate was significantly high in cathelicidin-alligator group at 81 %, followed by cathelicidin-sea snake (68 %), pleurocidin (50 %), cecropin (23 %) and infected control (17 %) (Figure 3B). None of the non-infected control had mortalities until challenge termination (data not shown). Cathelicidins from alligator and sea snake can significantly enhanced the survival rate of channel and hybrid catfish following *A. hydrophila* challenge.

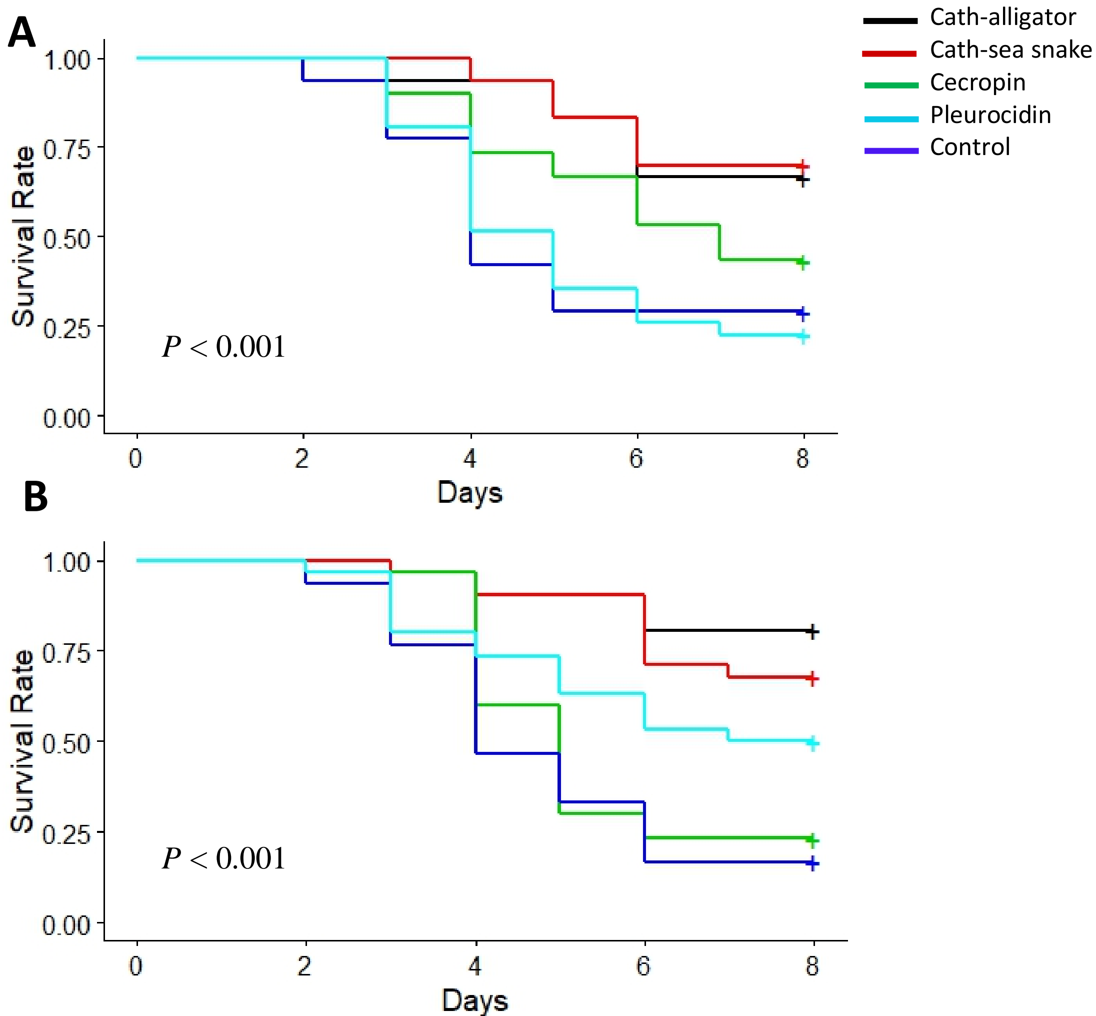

Figure 2 Trends for the survival rate of (**A**) channel catfish (*Ictalurus punctatus*) and (**B**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) injected with the different antimicrobial peptides, cathelicidin from alligator (cath-alligator), cathelicidin from sea snake (cath-sea snake), cecropin, and pleurocidin, and simultaneously infected with *Aeromonas hydrophila*. Peptides were intraperitoneally injected into fish (50 µg/mL), while control group were injected with saline only. Survival curves for channel catfish and hybrid catfish for all treatments and control were compared using the Kaplan-Meier test. The *P* values are for the Log Rank test which

indicates significant differences on survival curves between the different treatments for (**A**) channel catfish and (**B**) hybrid catfish.

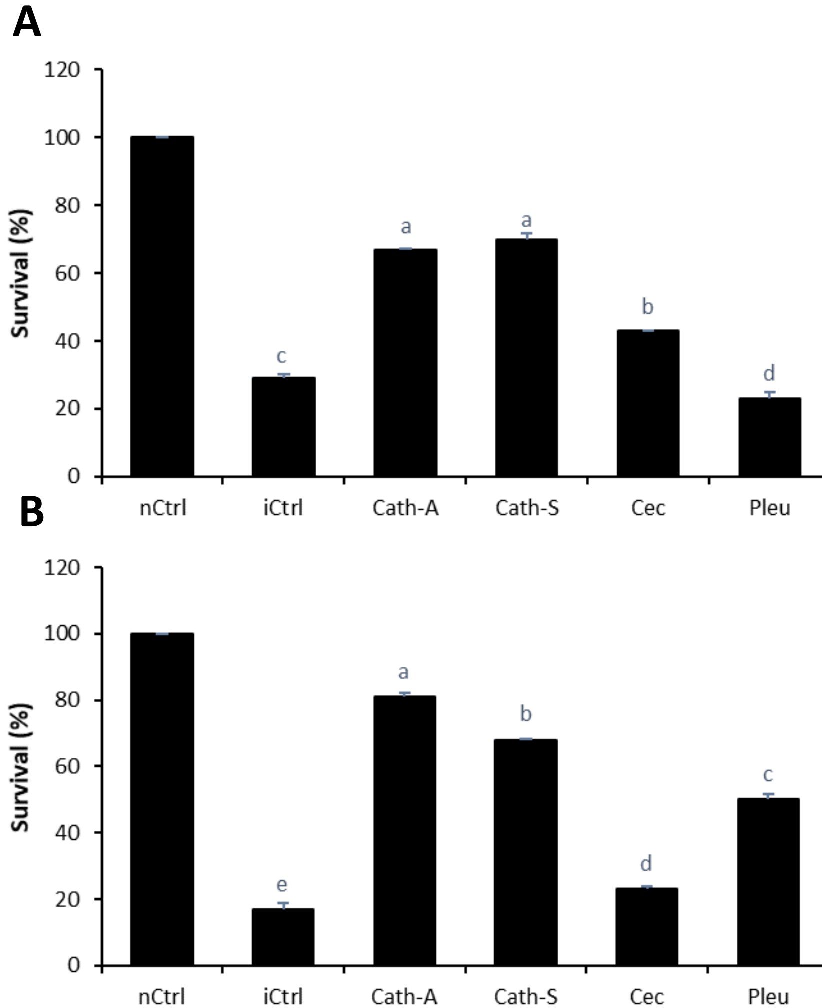

Figure 3 Effect of different antimicrobial peptides on the survival of *Aeromonas hydrophila*-infected channel catfish (*Ictalurus punctatus*) (**A**) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) (**B**). Peptides were intraperitoneally injected into fish (50 µg/mL) and challenged with *A. hydrophila* at 1 x 10^7 cells/mL. Peptide treatment groups include cathelicidin-alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with saline only (iCtrl)

while the non-infected controls (nCtrl) received no bacteria. Percent survival was analyzed by one-way ANOVA followed by Tukey's test. Means of survival with different letters are significantly different ($P < 0.01$).

Moreover, bacterial numbers in kidney and liver of channel and hybrid catfish for cathelicidin-injected groups decreased significantly after 24- and 48-h post-challenge infection (Figures 4 and 5). The bacterial numbers in fish that were injected with both cathelicidins were lower ($P < 0.05$) than the infected control and comparable to those fish that were not infected.

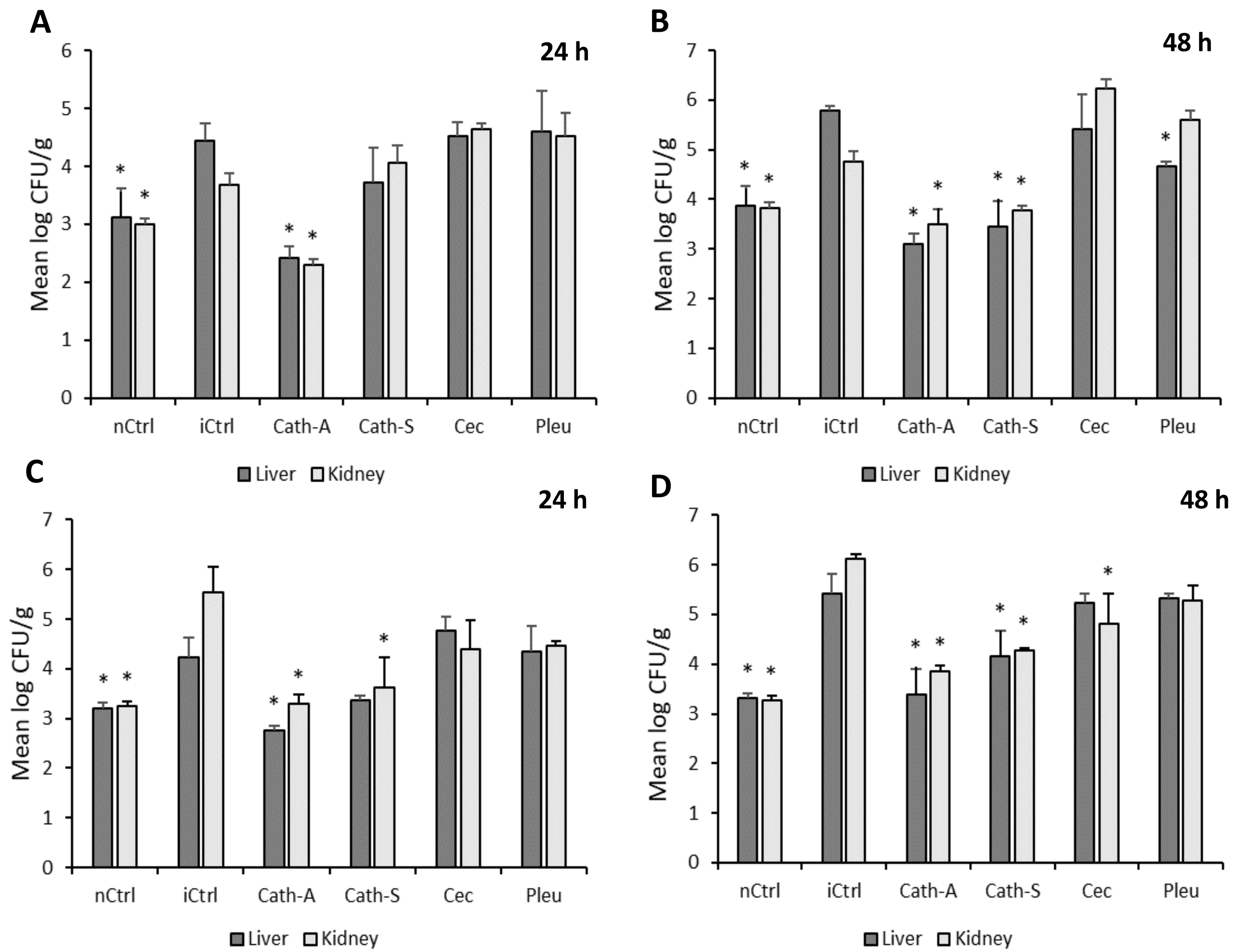

Figure 4 Effect of different antimicrobial peptides on bacterial numbers in liver and kidney of (**A**, **B**) channel catfish (*Ictalurus punctatus*) and (**C**, **D**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) infected with *Aeromonas hydrophila* at 24- and 48-h post infection. Peptide treatment groups include cathelicidin-alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with saline only (iCtrl) while the non-infected controls (nCtrl) received no bacteria. Mean log CFU/g were calculated from three fish for each treatment at each time point. Error bars indicate standard error (SEM). (*$P < 0.05$, significantly different with the infected control (iCtrl)).

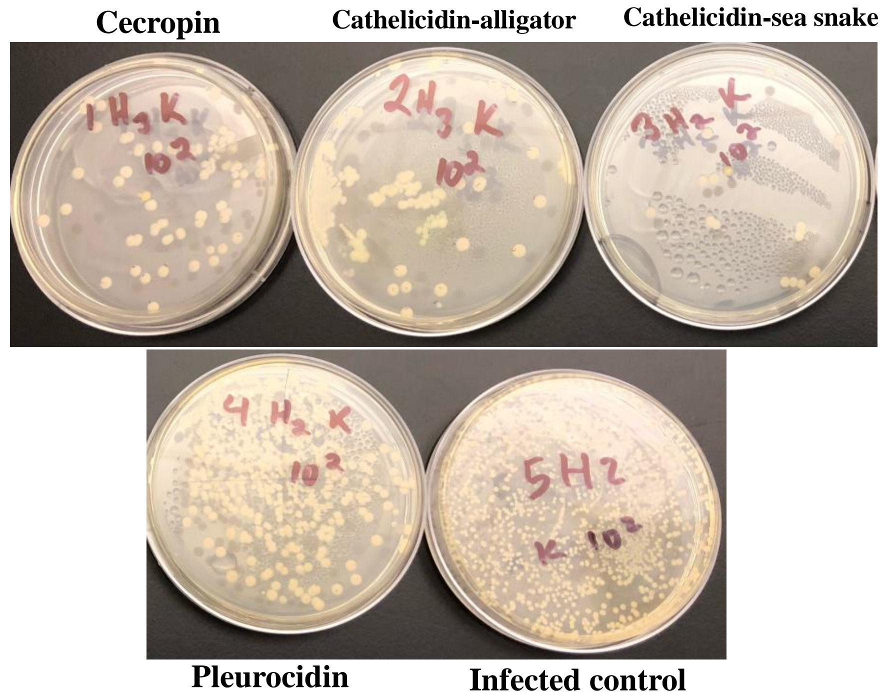

Figure 5 Representative plates of *Aeromonas hydrophila* colonies isolated from kidney samples of hybrid catfish (*Ictalurus punctatus* ♀ x blue catfish, *I. furcatus* ♂) 24 h post-infection with *Aeromonas hydrophila* in a bath immersion challenge. Hybrid catfish has been injected with different peptide treatments, cecropin, cathelicidin-alligator, cathelicidin-sea snake, and pleurocidin, and the infected control. Tissues were serially diluted with phosphate buffered saline (PBS), plated onto nutrient agar and incubated at 28°C for 18 h.

4. Discussion

The aquaculture industry experiences massive economic losses annually due to disease outbreaks. The catfish industry is plagued by bacterial diseases, which are the dominant type of disease. Some vaccines have been developed for important catfish pathogens namely *A. hydrophila*, *E. ictaluri* and *F. columnare* (Shoemaker et al., 2011; Zhang et al., 2014; Abdelhamed et al., 2018), but the high cost of developing vaccines and the labor intensiveness of the procedure sometimes negate economic benefits. Specifically, injectable vaccines are not an option for channel catfish because of the low value of individual fish. Finding an effective peptide that could inhibit the growth of these pathogens may present a unique opportunity to resolve bacterial disease problems in catfish by expressing these peptides via transgenesis or development of antibiotics. In fact, efforts have been made to utilize antimicrobial peptides as novel antibiotics, because of their unique modes of action against microorganism and do not easily induce resistance as compared to conventional antibiotics (Boman, 2003; Wang et al., 2008; Steinstraesser et al., 2011; Elad et al., 2012).

In vitro, high efficacy was observed for cathelicidin derived from alligator against five catfish pathogens. Alligator cathelicidin possesses potent antimicrobial activity against the five tested bacteria with MICs ranging from 6.25-100 µg/mL (Table 2). Antimicrobial peptides including defensins, cathelicidins and histatins have MICs in the range of 0.1-100 µg/mL (Bals, 2000). A recent study confirmed that cathelicidin from American alligator (AM-CATH36) has strong activity against multiple Gram-negative bacteria, including clinical isolates of multi-drug resistant (MDR) *Acinetobacter baumannii* and carbapenem-resistant *Klebsiella pneumoniae* using an MIC assay (Barksdale et al., 2017). Some studies have indicated that any AMP if tested at high concentrations *in vitro* (i.e. higher than physiologically relevant levels) can increase

membrane permeation (Shai, 1995; Shai, 2002). For example, Patrzykat et al. (2002) observed that a pleurocidin analog at the MIC rapidly inhibited RNA and protein synthesis in *Escherichia coli* without affecting the integrity of the bacterial membrane permeability barrier. But at 10X MIC, *E. coli* cells became polarized, indicating that the peptide disrupted the membranes at high levels. This implied that AMPs have multitarget mechanisms of action, affecting either RNA or DNA synthesis, cellular enzymes, the process of cell division or membranes (Hancock and Sahl , 2006). Further studies are warranted to investigate the exact mode of action of alligator cathelicidin in inhibiting the growth of most catfish pathogens *in vitro*.

A. hydrophila, an important catfish pathogen, was one of the bacteria that was susceptible to peptide inhibition by exhibiting the lowest MIC to most of the peptides tested, and was further selected for the killing kinetic assay. The two cathelicidins (from alligator and sea snake) had rapid antimicrobial effect comparable to ampicillin because the bacterial colonies decreased to zero after 120 min. Guo et al. (2017) also performed the same assay but used cathelicidin (As-CATH4 and 5) from Chinese alligator, *Alligator sinensis*, and found that As-CATH4 and 5 could decrease *Vibrio parahaemolyticus* bacterial colonies to zero within 90 min at 10X MIC. Usually, drug-resistance is unavoidable among bacteria after long-time treatment. However, for small amino peptides, antimicrobial molecules kill too quickly for the bacteria to undergo induction of drug-resistance. Thus, peptides with rapid antimicrobial activities are less likely to induce bacterial resistance as compared to others with slow antimicrobial effects (Kosciuczuk et al., 2012).

Two major mechanisms of action have been proposed to explain the membrane-disruptive activity of cathelicidin, like that of other cationic AMPs (Kosciuczuk et al., 2012; Lee et al., 2016). One is that the peptides aggregate near the bacterium, then insert into the

membrane creating a channel or the barrel-stave model, and the other is the carpet model where the hydrophobic surfaces of the peptides associate with the bacterial membrane, causing it to become thin until pores are formed (Shai, 2002; Brogden, 2005; Kosciuczuk et al., 2012). Cathelicidins are particularly effective against Gram-negative bacteria as confirmed by this study and others (Wang et al., 2008; Barksdale et al., 2017; Guo, et al., 2017). This can be due to the interaction of cationic peptide with the negatively charged lipid membranes of microorganisms allowing them to move across the bacterial outer membrane, passing the layer of peptidoglycan, and finally crossing the inner membrane into the cytoplasm of the bacterial cell (Reddy et al., 2004).

Interestingly, cathelicidins are less cytotoxic to host cell membranes. Barksdale et al. (2017) conducted hemolysis assays and found that cathelicidin from alligator was not hemolytic against sheep red blood cells at 300 mg/mL as well as not cytotoxic towards A549 human lung epithelial cells after 24 h exposure. Similarly, cathelicidin from sea snake (Hc-CATH) even at high concentration of 200 μg/mL exhibited very low cytotoxicity towards various human cell lines (HepG2, PC3 and L929) and mouse peritoneal macrophage cells (Wei et al. 2015). This may be due to cell selectivity of AMPs, i.e. they selectively inhibit microorganisms without being significantly toxic to host cells (Matsuzaki, 2009). The molecular basis for this cell selectivity is the cationic property of AMPs which makes it more attracted to the negatively charged membranes of bacterial cells, thereby exerting its antimicrobial activity. The absence of charge attraction between the cationic peptides and the neutral cell surface of mammalian cells suggests that it should be safe for future therapeutic use (Duwadi et al., 2018).

The interaction between cationic peptide and anionic microbial cell membrane can be influenced by salt concentrations in the solution. For instance, the cathelicidin identified in

sheep and goat, Bac5, was able to inhibit all Gram-negative bacteria tested at salt concentrations similar to those found in extracellular fluids (Shamova et al., 1999). At low salt concentration (10 mM NaCl), Bac5 showed high activity against the microbes studied, whereas, at high salt concentration (100 mM NaCl) peptides are still active against Gram-negative bacteria such as *E. coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa*, but have no activity against *Staphylococcus aureus* and *Candida albicans* (Shamova et al., 1999). But the antimicrobial potency of some AMPs was not significantly impacted by the concentration of salt in the media. Zhao et al. (2008) observed that the OH-CATH peptide from king cobra, *Ophiophagus hannah*, showed greater potency than the human cathelicidin LL-37 against a variety of known human bacterial pathogens but its antimicrobial activity was not affected by salt concentrations. Even the smaller peptide fragments of OH-CATH were found to be active both *in vitro* and *in vivo* against bacterial isolates tested including multi-drug resistant strains (Zhang et al., 2010; Li et al., 2012). Many of the genes annotated in alligator cathelicidins are very similar to the snake cathelcidins (e.g. OH-CATH) (van Hoek, 2014), which may partly explain the effectiveness of alligator cathelicidin in inhibiting bacterial growth both *in vitro* and *in vivo* as observed in this study. Although the effect of salt concentration on the antimicrobial activity of alligator cathelicidin was not determined, it was hypothesized that under normal physiological conditions in the body of catfish, alligator cathelicidin peptide injection (50 µg/mL) seemed to exert its antimicrobial effects *in vivo* following vAh infection.

Previous experimental challenge models on the effect of vAh in catfish have achieved variable results. Some challenge protocols were characterized by low and variable mortality of 0-20 % (Zhang et al., 2016), while others have achieved mass mortality <24 h after infection, mimicking the natural outbreaks in most catfish farms (Pridgeon et al., 2011; Pridgeon and

Klesius, 2011). While a 90 % mortality within 48 h was observed by Zhang et al. (2016) on adipose fin clipped channel catfish challenged with vAh. In this study, the fish were also adipose fin-clipped after peptide injection. The infected control started dying 48 h post infection for both channel catfish and hybrid catfish, with accumulated mortalities of 71 and 83 % for channel catfish and hybrid catfish, respectively, at the end of the challenge period. Most of the fish injected with the peptides started dying at day 3, with pleurocidin having the highest mortality for channel catfish and cecropin for hybrid catfish. Consistent with the *in vitro* results, the two cathelicidins enhanced protection for both channel catfish and hybrid catfish against vAh infection with highest survival rates ranging from 67-81% as compared to other peptides and infected control. These results were in agreement with Guo et al. (2017) who also found increased survival rates in crabs injected with cathelicidin peptides, As-CATH4 and 5 following *A. hydrophila* infection. Furthermore, the decrease in bacterial numbers specifically in fish injected with cathelicidin-alligator (Figure 4) suggested that it can provide both channel catfish and hybrid catfish with the capability to eliminate invading bacteria.

Differences in the infection pattern between channel catfish and hybrid catfish were observed in this study. The hybrid catfish infected control had lower survival rate (17%) than the channel catfish infected control (29%) (Figure 3). In previous studies, hybrid catfish was observed to be more resistant than channel catfish to *A. hydrophila* (Dunham and Masser, 2012), while blue catfish was noted to be more resistant to vAh than either channel catfish or channel catfish x blue hybrid catfish (Li et al., 2013). These variations may be due to host's innate resistance, and physiological and immunological status at the time of exposure to the pathogen, strain and family differences, mode of infection and environment. Detailed analysis of variations among various channel catfish, blue catfish and hybrid catfish have not yet been elucidated, as

there might be complex regulatory factors involved between activation and inhibition to ensure the elimination of invading pathogens as well as the protection from the host's own defense reactions (Rebl and Goldammer, 2018). Nevertheless, hybrid catfish injected with both alligator and sea snake cathelicidins have higher survival rates than cathelicidin-injected channel catfish. It seemed that cathelicidins worked better in hybrid catfish for conferring protection from vAh infection.

5. Conclusion

Screening for a potent antimicrobial peptide might be an effective approach to lead to addressing bacterial disease problems in the catfish industry. Cathelicidins derived from alligator and sea snake possessed rapid antimicrobial activities against major catfish pathogens *in vitro*. Both cathelicidins also enhanced protection of channel catfish and hybrid catfish from virulent *A. hydrophila* immersion challenge. Therefore, there is a great prospect in the development of disease-resistant fish through ectopic expression of genes encoding these peptides.

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CHAPTER THREE

Cathelicidins enhance protection of channel and channel-blue hybrid catfish against *Edwarsiella ictaluri* infections

Abstract

Cathelicidins are a class of antimicrobial peptides (AMPs) known to possess rapid and direct antimicrobial activities against a variety of microorganisms. Recently identified cathelicidins derived from alligator and sea snake were found to be more effective in inhibiting microbial growth and induce immune modulating activities than other AMPs previously characterized. The ability of these two cathelicidins along with the peptides, cecropin and pleurocidin, to protect channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) against *Edwardsiella ictaluri*, one of the most prevalent pathogen affecting commercial catfish industry, was investigated. Cathelicidin-injected fish (50 µg/mL) that were simultaneously challenged with *E. ictaluri* through bath immersion at a concentration of ~1 x 10^6 cells/mL had increased survival rates compared to other peptide treatments and the infected control. Bacterial numbers were also reduced in the liver and kidney of channel catfish and hybrid catfish in the cathelicidin treatments 24 h post-infection. After 8 days of challenge, serum was collected to determine immune-related parameters such as bactericidal activity, lysozyme, serum protein, albumin and globulin. These immune-related parameters were significantly and consistently elevated in fish injected with the two cathelicidins as compared to other peptides and the infected control. These results indicate that cathelicidins derived from alligator and sea snake can stimulate immunity and enhance the resistance to *E. ictaluri* infection in channel catfish and hybrid catfish.

1. Introduction

Edwardsiella ictaluri is the causative agent of enteric septicemia of catfish (ESC), one of the most prevalent infectious disease in the commercial catfish industry in the United States (Hawke et al., 1981; Hawke, 2015). *E. ictaluri* is a Gram-negative facultatively anaerobic motile rod, that grows at 28-30°C (Plumb and Sanchez, 1983). It enters catfish through the intestinal tract, the nares, the gills, and the skin (Menanteau-Ledouble et al., 2011; Hawke et al., 2015) and is incorporated in leukocytes, particularly in macrophage (Shotts et al., 1986; Baldwin and Newton, 1993). The acute form of the disease causes gastroenteric septicemia, and its chronic form can result in meningitis or meningoencephalitis (Hawke et al., 2015). The late stages of this disease cause swelling and ulceration on the dorsum of the head, exposing the brain, which has led to the term "hole in the head disease", used in the industry (Morrison and Plumb, 1994). ESC occurs predominantly at a temperature range between 20 to 28°C with highest losses in heavily stocked ponds that have experienced environmental stressors within this temperature range (Hawke et al., 2015). Acute outbreaks of ESC can cause losses of over 50 % in an infected population, leading to economic losses of $40–60 million in the US catfish industry annually (Shoemaker et al., 2009).

Resistance to ESC greatly varies among different catfish species. Channel catfish (*Ictalurus punctatus*) is highly susceptible to ESC infection, while blue catfish (*I. furcatus*) is very resistant (Wolters and Johnson 1994; Wolters et al. 1996). Furthermore, Wolters et al. (1996) observed that the survival and antibody response in hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) demonstrated greater ESC resistance than in channel catfish. Production of channel-blue hybrid catfish is becoming popular in commercial farms due to several advantages such as faster growth, increased disease resistance, high fillet yield, increased

tolerance to low oxygen and increased vulnerability to angling and seining (Dunham and Smitherman, 1987; Dunham et al., 2008). Recently, a genome-wide association study (GWAS) was conducted by Tan et al. (2018) on interspecific backcross (channel catfish ♀ x F1 channel-blue hybrid catfish ♂) progenies and found that strong resistance alleles against ESC originated from blue catfish. Studies such as this could provide understanding of the genetic basis of desirable traits, particularly the specific location of quantitative trait loci (QTL) affecting the traits.

Effective approaches to controlling infectious aquaculture diseases are highly desirable. Substantial evidence suggests that endogenous peptides with antimicrobial properties can result to increased resistance to bacterial pathogens in transgenic animals (Reed et al., 1997; Dunham et al., 2002; Chiou et al., 2014). Most of these antimicrobial peptides (AMPs) share common characteristics such as small size, cationic charge, amphipathic character and display broad-spectrum antimicrobial activity (Hancock and Sahl, 2006). They are located on epithelial surfaces where they function as first line of defense against microbial invasion, and in circulating myeloid-derived cells which accumulate during infection (Cole et al., 2000; Chang et al., 2005). These peptides are classified into several families, including the widely studied molecules such as cecropins from insects, magainins from amphibians, and defensins and cathelicidins from mammals (Boman, 1995).

Recently, a great deal of interest was focused on cathelicidins because they possess direct and antimicrobial activities against a variety of microorganisms, such as bacteria, fungi, virus and parasites. Since its first discovery in bovine neutrophils, many cathelicidins were also identified in other vertebrates (Bals and Wilson, 2003; Kosciuczuk et al., 2012). Additional important functions of cathelicidins involved host immune modulation and disease resistance,

including promotion of angiogenesis and wound healing (Wong et al., 2013). Moreover, recent studies confirmed that their effectiveness to kill microbes and immune modulating activities are more potent than other AMPs previously characterized. For instance, Wei et al. (2015) identified and characterized the first cathelicidin from sea snake, *Hydrophis cyanocinctus* and found that it can induce microbial membrane permeabilization resulting in cellular disruption of both Gram-positive and Gram-negative bacteria. Novel cathelicidins from American alligator (*Alligator mississippiensis*) (Barksdale et al., 2017) and Chinese alligator (*A. sinensis*) (Chen et al., 2017) were also found to possess potent antimicrobial and immunomodulatory properties while not being cytotoxic towards mammalian cells.

Thus, we sought to investigate the effectiveness of cathelicidins in protecting channel catfish and hybrid catfish from *E. ictaluri* infection. The immune stimulation effect of cathelicidins from alligator and sea snake was compared to cecropin and pleurocidin.

2. Materials and Methods

2.1. Experimental fish and bacteria

Channel catfish and hybrid catfish (mean ± SD weight 10.60 ± 3.40 g) were obtained from stocks maintained at the Auburn University Fish Genetics Laboratory and acclimated in dechlorinated tap water at 25-28°C for 48 h prior to experiment. Fish were placed in a 365-L tank (height, width and length were 57 cm, 72 cm and 3 m, respectively) for acclimation with continuous aeration and water flow. Throughout the duration of the study, water temperature was 28.0 ± 0.4°C, pH was 7.0 ± 0.2, dissolved oxygen was 6.50 ± 0.50 mg/L, nitrite was 0.50 ± 0.2 mg/L, alkalinity was 80 mg/L and ammonia concentration was 0.50 ± 0.05 mg/L. Fish

handling and treatment procedures used during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC).

E. ictaluri strain S97-773 was provided by the USDA-ARS, Aquatic Animal Health Research Unit, Auburn, AL. A frozen glycerol stock of *E. ictaluri* was inoculated into 20 mL brain heart infusion broth (BHIB; BD Difco, Sparks, Maryland) and grown at 26°C in a shaker incubator at 180 rpm for 24 h. Cultures were then expanded into 1 L BHIB and grown under the same conditions for 24 h until the cell density reached to approximately 1 x 10^8 cells/mL based on an absorbance value read at 600 nm (OD_{600}). BHIB was inoculated with the iron chelator (xenosiderophore) deferoxamine mesylate (DFO; Sigma, St. Louis, MO, USA) at a final concentration of 0.2 mM (Galet et al., 2015) to further promote bacterial virulence. Standard plate counts in triplicates were carried out to enumerate the average number of colony forming units per milliliter (CFU/mL) of bacterial cells used to challenge fish.

2.2. Synthesis of peptides

The peptides used in this study (Table 4) were custom ordered from Biomatik Corporation (Cambridge, Ontario, Canada). The synthetic peptides were purified and analyzed by HPLC and Mass Spectrometry to confirm purity of greater than 95 % (Biomatik). Peptides were dissolved in distilled water at 1 mg/mL as stock solution before use.

Table 4 Peptide sequences used in intraperitoneal injection of channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂), then challenged with *Edwardsiella ictaluri.*

Peptide	Sequence	Reference
Cecropin B	KWKVFKKIEKMGRNIRNGIVKAGPAIAVLGEAKAL	Dunham et al. 2002
Cathelicidin alligator	GLFKKLRRKIKKGFKKIFKRLPPIGVGVSIPLAGKR	Barksdale et al. 2017
Cathelicidin sea snake	KFFKRLLKSVRRAVKKFRKKPRLIGLSTLL	Wei et al. 2015
Pleurocidin amide	GWGSFFKKAAHVGKHVGKAALTHYL-NH_2	Jia et al. 2000

2.3. Bacterial challenge

Fish used for the challenge were anesthetized with 0.01 % tricaine methanesulfonate (MS-222), buffered with 0.02 % sodium bicarbonate, pH 7.35 to 7.45). The peptides were delivered by intraperitoneal injection of a stock at a concentration of 50 µg/mL. Control fish were injected with saline only. There were four peptide treatment groups: cecropin B, cathelicidin-alligator, cathelicidin-sea snake and pleurocidin amide. Each treatment group consisted of three replicates with 10 fish per basket. The wire mesh baskets were labelled with codes and were arranged in a randomized block design. There were two types of control used in this study: infected and non-infected. The infected control group was placed in the infected tank with the peptide treatments, while the non-infected control group was in a separate tank and received only BHIB. Before the start of the challenge, water was lowered in the tank to 100 L total volume. One liter of *E. ictaluri* bacterial suspension containing approximately $1.0 \pm 0.5 \times 10^8$ cells was added to the tank resulting in a final concentration of $\sim 1.0 \pm 0.5 \times 10^6$ cells/mL. Fish were immersed statically for 1 h with aeration. At the conclusion of 1 h static challenge,

water was restored. The infected control group, which received saline only, were placed together in one tank with the peptide group, while the non-infected control group was in a separate tank and received no bacteria only BHIB. Fish were observed every 4 h and dead or moribund fish were either sampled or removed. To confirm the presence of *E. ictaluri* as the causative agent of mortality, dead or moribund fish were sampled to isolate bacteria from kidney and liver. Fish were not fed on the day of the challenge but were given food *ad libitum* the next day after the infection and throughout the experiment.

2.4. Bacterial counts in fish kidney and liver

After 24 h post infection, five fish from each treatment were randomly collected, anesthetized and had their kidneys and livers removed. The tissues were immediately diluted (1000 x) with sterile phosphate buffered saline (PBS) buffer and homogenized. Fifty microliters of the tissue dilutions were plated on nutrient agar and the plates incubated at 28°C for 24-48 h. The number of viable bacterial colonies grown on the plates was recorded and expressed as log colony forming units/gram (CFU/g).

2.5. Blood sampling

Five fish per treatment were sampled at the end of the challenge period (day 8). Blood was withdrawn by caudal venipuncture using a 22-gauge needle with syringe. Serum was obtained by allowing the blood to clot overnight at 4°C. Then, serum was collected by centrifuging at 15,000 *x g* for 5 min and stored at -20°C until used.

2.6. Serum bactericidal assay

Serum bactericidal assays can give information on whether a given serum has a level of bactericidal antibodies sufficient to reach a protective threshold and can be evaluated using a traditional viable count technique. Bacterial culture of *E. ictaluri* was centrifuged and the pellet

washed and suspended in 1 x PBS. Optical density (OD) of the bacterial suspension was adjusted to 0.2 at 546 nm, then serially diluted (1:10) with PBS five times. To determine serum bactericidal activity, 2 µL of this diluted bacterial suspension was incubated with 20 mL serum in a micro vial for 1 h at 28°C. PBS replaced the serum in the bacteria control group. The number of viable bacteria was determined by counting the colonies grown on a brain heart infusion agar plate for 24-48 h at 28°C (Aly et al., 2008).

2.7. Lysozyme assay

Lysozyme assay is based on the ability of the enzyme lysozyme to cleave a synthetic substrate and release a free flourophore which can be easily quantified. Bacterial cell wall lysis has been widely employed as a measure of lysozyme activity. Serum lysozyme activity was determined using a diagnostic kit (Catalog no. LY0100, Sigma, St. Louis, MO, USA). It is based on the lysis of lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* by the lysozyme present in the serum. During incubation of the lysozyme sample and substrate, the reaction is followed by monitoring the decrease in absorbance at 450 nm. The rates of reduction in absorbance of samples was converted to lysozyme concentration (units/mL) using the standard curve.

2.8. Determination of serum protein, albumin and globulin

Following the increase in bactericidal proteins, serum biochemical parameters such as protein, albumin and globulin were measured to determine the increase in protective protein production after a bacterial infection. Total serum protein and albumin were determined using a diagnostic kit (Catalog nos. TP0100 and MAK124, respectively, Sigma). For globulin levels, albumin was subtracted from the total protein. The protein concentrations of the samples were expressed as mg/mL serum.

2.9. Statistical analysis

Data were analyzed using one-way ANOVA and Tukey's multiple comparisons test to determine differences between treatment means. Survival curves for channel catfish and hybrid catfish injected with different peptides and control groups were compared using the Kaplan-Meier test (Goel et al., 2010). Comparison of survival curves of all the treatments were performed using the Log Rank test (Bland and Altman, 2004). *P*-values of 0.05 or less were considered statistically significant. The Welch *t*-test was used to compare survival rates between channel catfish and hybrid catfish. Analyses were performed using R software (R Core Team, 2014) and figures were made in Microsoft Excel 2016 and GraphPad Prism 8 (GraphPad Software, San Diego, CA).

3. Results

3.1. Resistance against *E. ictaluri* infection

After peptide injection into channel catfish and hybrid catfish and bacterial challenge, mortality was recorded for 8 days. Channel catfish with different peptide treatments and the infected control except for cathelicidin-sea snake started dying at day 3 of the challenge (Figure 6A). While in hybrid catfish (Figure 6B), treatment groups, cathelicidin-alligator and pleurocidin started dying early at day 2. Survival rate was higher in the cathelicidin-sea snake treatment (47 %) as compared to other treatments ($P < 0.01$) (Figure 7A). In hybrid catfish, survival rate was highest in cathelicidin-alligator group at 53 %, followed by cecropin (50 %), pleurocidin (27 %), infected control (23 %) and cathelicidin-sea snake (17 %) ($P < 0.01$) (Figure 7B). The non-infected control had no mortalities until the end of the challenge period. All dead

fish exhibited symptoms of *E. ictaluri* infection such as petechial hemorrhaging on the ventral surface, swollen abdomen, and more.

Furthermore, bacterial numbers in kidney and liver of channel catfish and hybrid catfish injected with cathelicidins decreased significantly after 24 h post-challenge infection (Figure 8). The bacterial numbers in fish that were injected with either of cathelicidins had consistently lower bacterial counts ($P < 0.05$ and $P < 0.01$) than other peptides and the infected control. Although the results were variable between channel catfish and hybrid catfish, overall, cathelicidins from alligator and sea snake significantly enhanced the survival rate of channel catfish and hybrid catfish following *E. ictaluri* challenge.

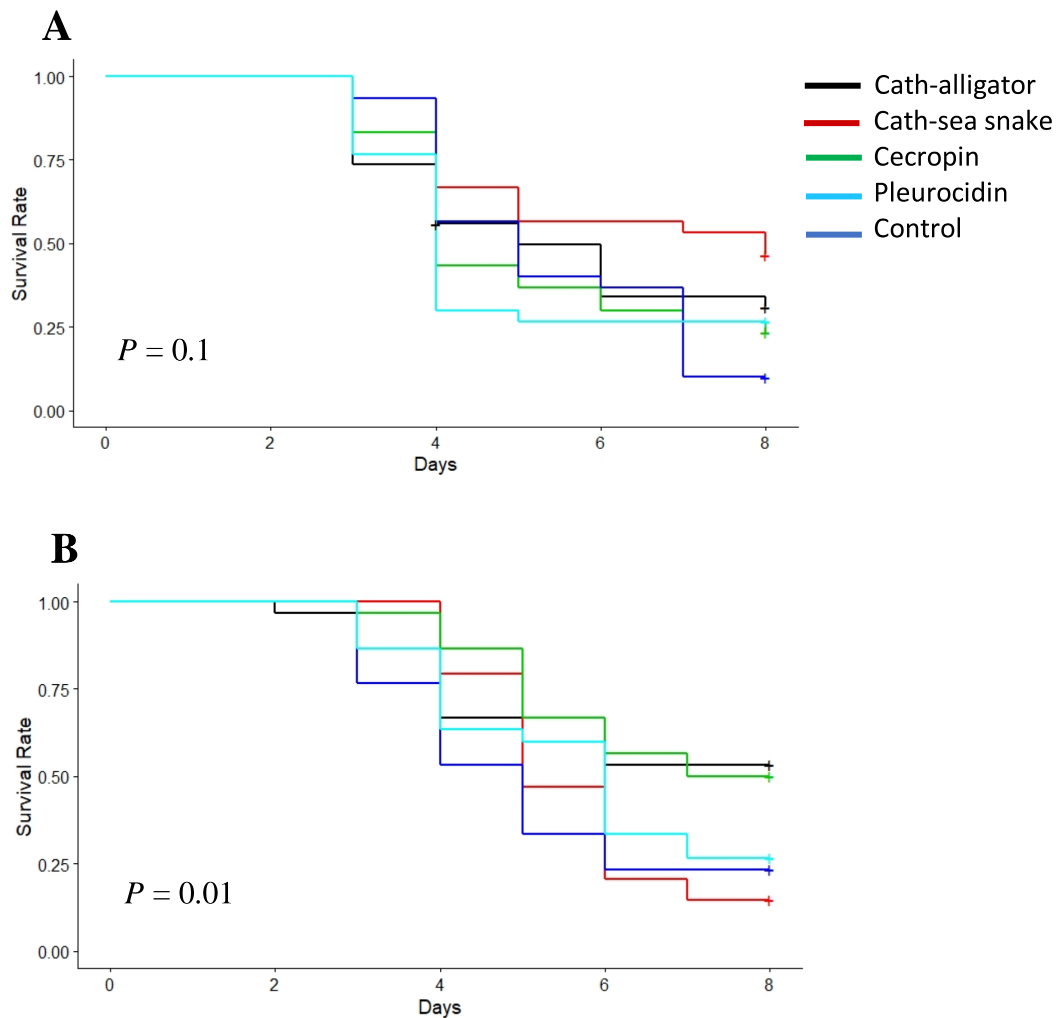

Figure 6 Trends for the survival rate of (**A**) channel catfish (*Ictalurus punctatus*) and (**B**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) injected with the different antimicrobial peptides, cathelicidin from alligator (cath-alligator), cathelicidin from sea snake (cath-sea snake), cecropin, and pleurocidin, and simultaneously infected with *Edwardsiella ictaluri*. Peptides were intraperitoneally injected into fish (50 µg/mL), while control group were injected with saline only. Survival curves for channel catfish and hybrid catfish for all treatments and control were compared using the Kaplan-Meier test. The *P* values are for the Log Rank test which

indicates significant differences on survival curves between the different treatments for (**A**) channel catfish and (**B**) hybrid catfish.

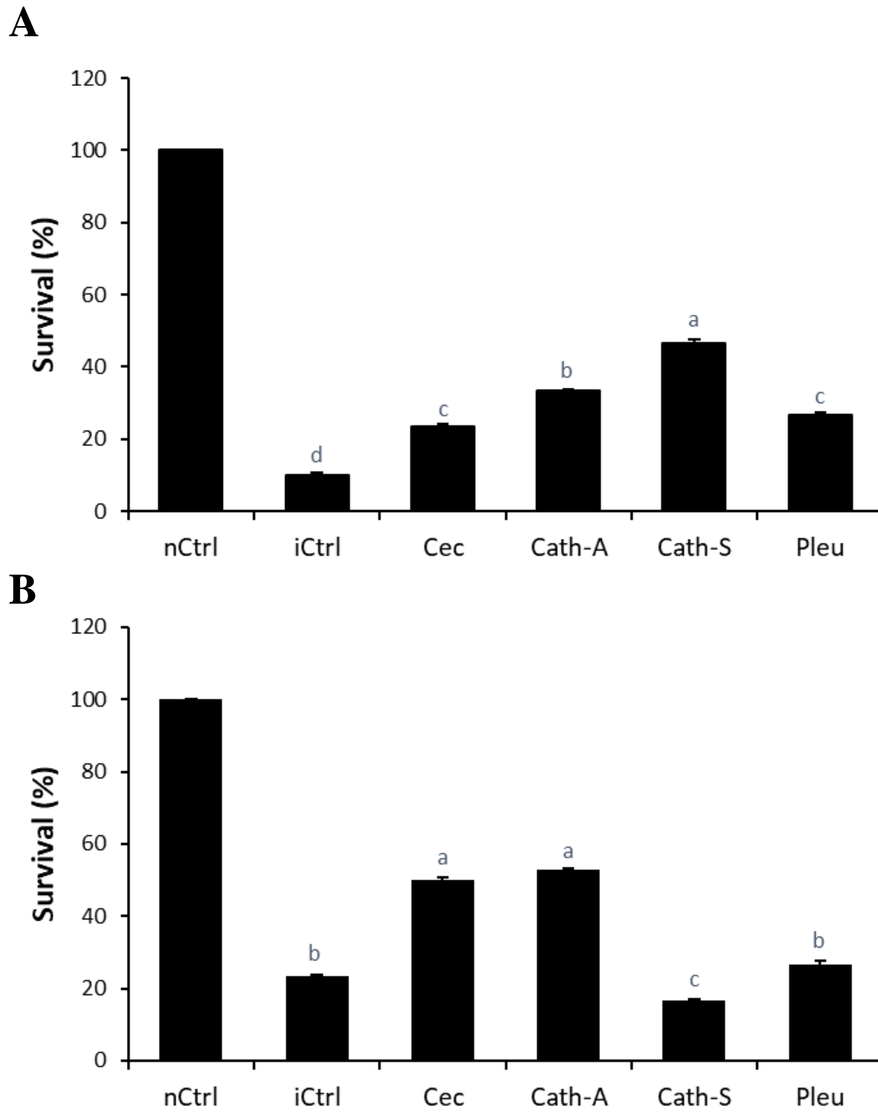

Figure 7 Effect of different antimicrobial peptides on the survival of (**A**) channel catfish (*Ictalurus punctatus*) and (**B**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) infected with *Edwardsiella ictaluri.* Peptides were intraperitoneally injected into fish (50 µg/mL) and challenged with *E. ictaluri* at ~1 x 10^6 cells/mL. Peptide treatment groups include cathelicidin-

alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with saline only (iCtrl) while the non-infected controls (nCtrl) received no bacteria. Percent survival was analyzed by one-way ANOVA followed by Tukey's test. Means of survival with different letters are significantly different ($P < 0.01$).

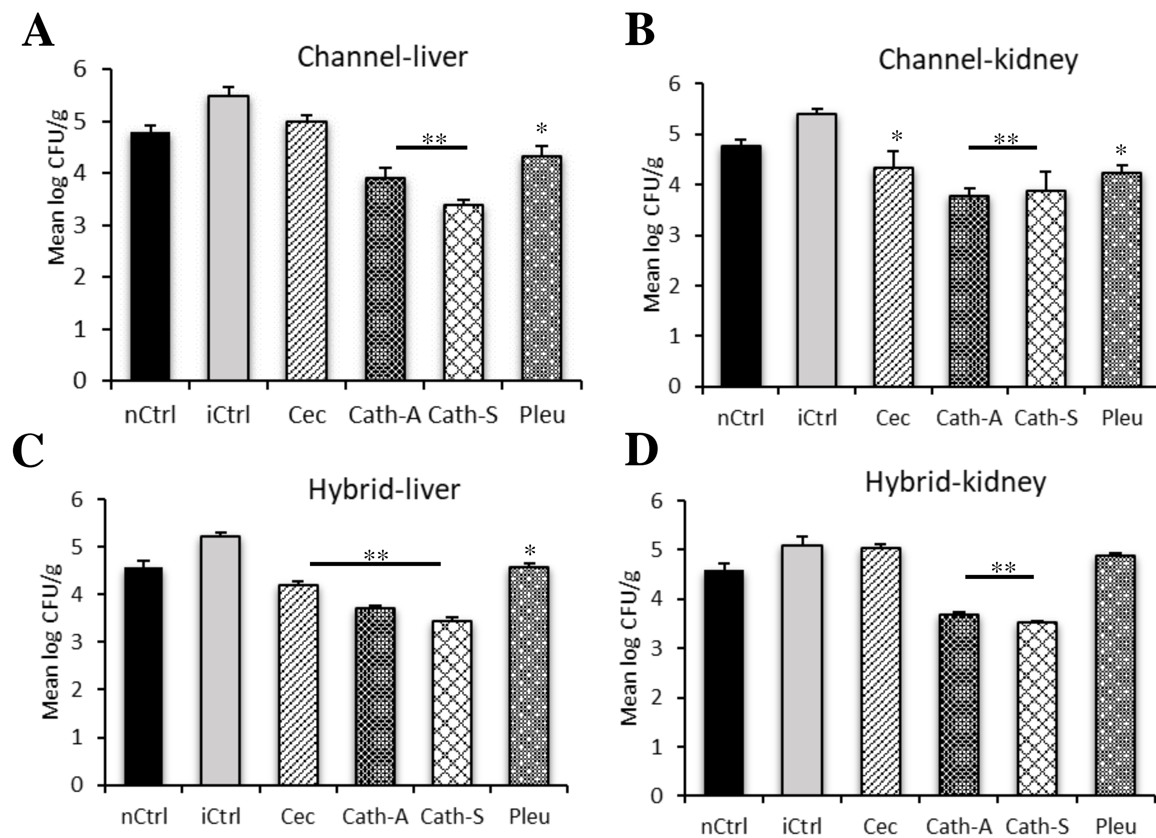

Figure 8 Effect of different antimicrobial peptides on bacterial numbers in liver and kidney of (**A**, **B**) channel catfish (*Ictalurus punctatus*) and (**C**, **D**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) infected with *Edwardsiella ictaluri* at 24 h post infection. Peptide treatment groups include cathelicidin-alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with

saline only (iCtrl) while the non-infected controls (nCtrl) received no bacteria. Mean log CFU/g were calculated from five fish for each treatment and analyzed by one-way ANOVA followed by Tukey's test. (*$P < 0.05$, ** $P < 0.01$ significantly different from the infected control (iCtrl)).

3.2. Serum bactericidal activity

The viable bacterial counts were lower ($P < 0.01$) in both cathelicidins (alligator and sea snake) as compared to other peptides, infected control and non-infected control (Figure 4). In the channel catfish group (Figure 9A), the highest number of bacterial colonies were counted in the non-infected and infected control (269 and 257 colonies, respectively), followed by cecropin (36 colonies), pleurocidin (20 colonies), cathelicidin sea snake (16 colonies), and cathelicidin alligator (10 colonies). While in the hybrid catfish group (Figure 9B), pleurocidin had the highest bacterial counts (231 colonies) while cathelicidin alligator had the lowest at 75 colonies ($P < 0.01$). Fewer bacterial colonies were seen in plates treated with cathelicidin alligator in both channel catfish and hybrid catfish groups (Figure 10).

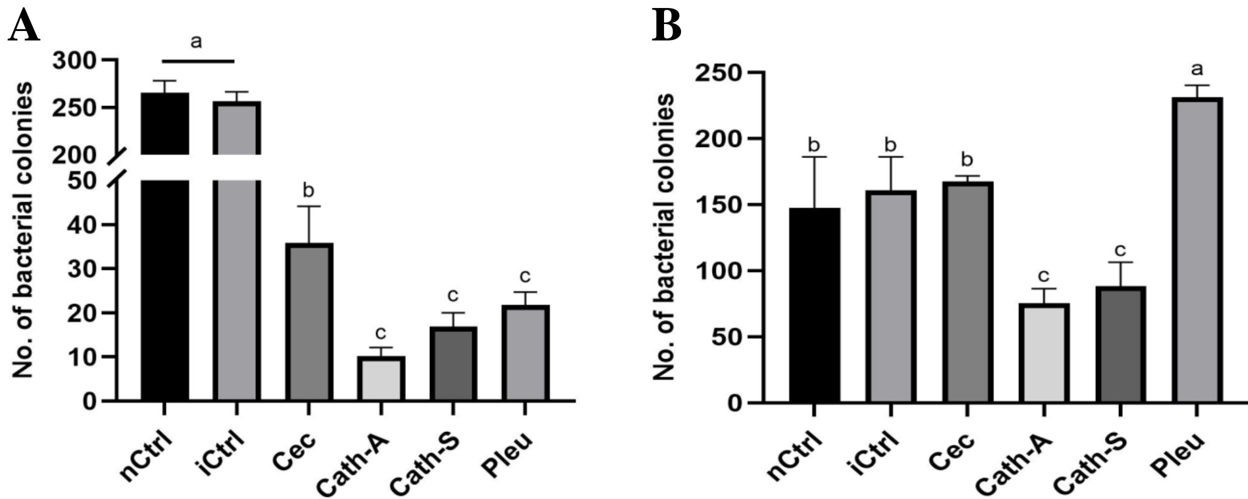

Figure 9 Effect of different antimicrobial peptides on serum bactericidal activity of (**A**) channel catfish (*Ictalurus punctatus*) and (**B**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) infected with *Edwardsiella ictaluri.* Peptide treatment groups include cathelicidin-alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with saline only (iCtrl) while the non-infected controls (nCtrl) received no bacteria. The values represent mean ± SEM of five fish and analyzed by one-way ANOVA followed by Tukey's test. Means with different letters are significantly different ($P < 0.01$).

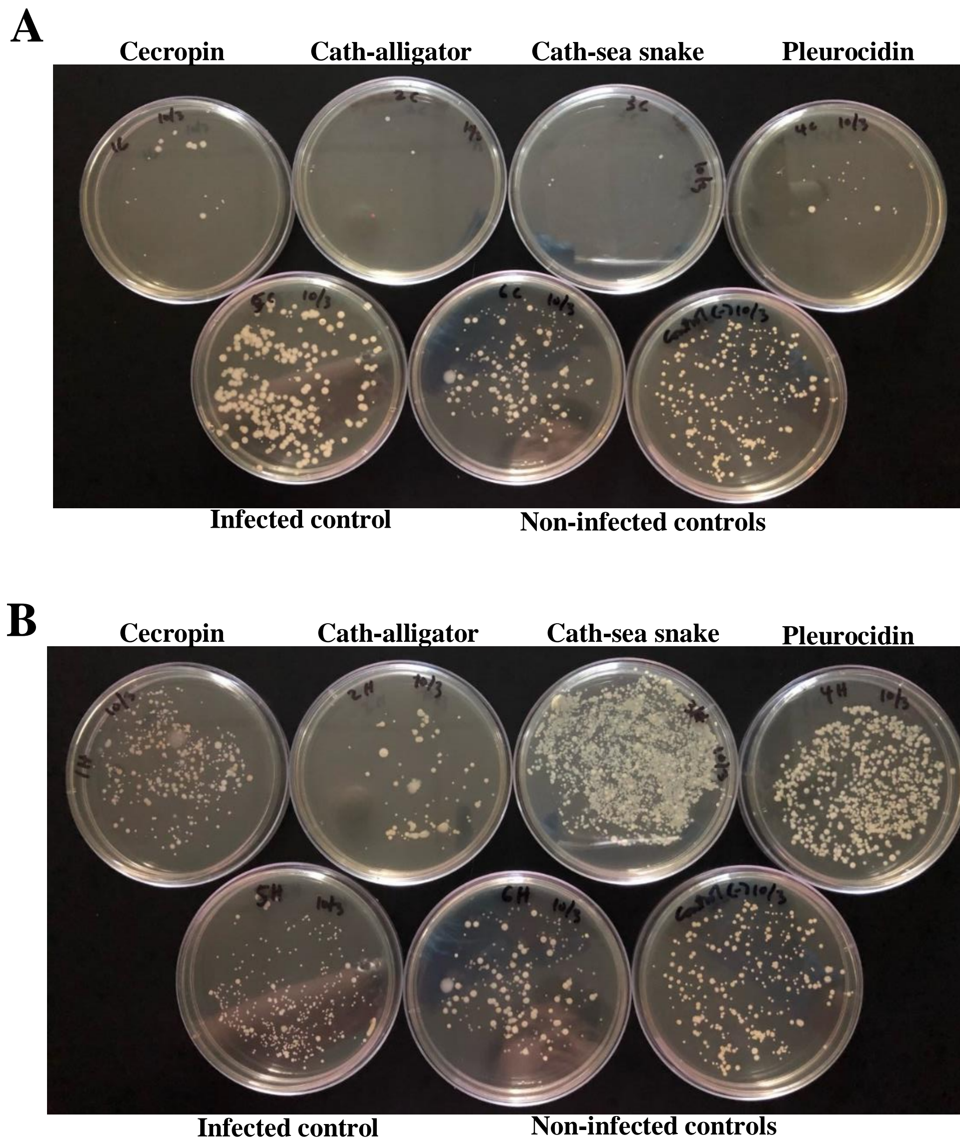

Figure 10 Representative plates of *Edwardsiella ictaluri* colonies from serum bactericidal assay of (**A**) channel catfish (*Ictalurus punctatus*) and (**B**) hybrid catfish (*Ictalurus punctatus* ♀ x blue catfish, *I. furcatus* ♂). Sera from each treatment were collected and incubated with diluted bacterial suspension of *E. ictaluri* for 1 h. Phosphate buffered saline (PBS) replaced the serum in the bacteria control groups. Colonies were grown on nutrient agar plate for 24-48 h at 28°C.

3.3. Serum lysozyme levels

High levels of serum lysozyme were found in cathelicidin-alligator (78 units/mL) and cathelicidin-sea snake (89 units/mL) in channel catfish and hybrid catfish groups, respectively, compared to other peptides and controls ($P < 0.05$) (Figure 11). Consistently, the lowest lysozyme levels were found in the infected control groups for both channel catfish and hybrid catfish.

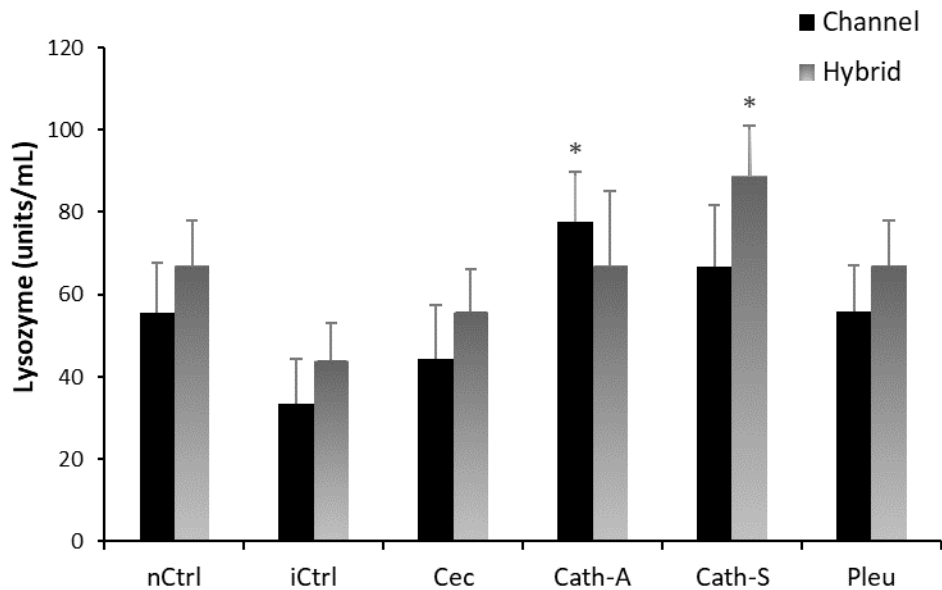

Figure 11 Effect of different antimicrobial peptides on serum lysozyme levels of channel catfish (*Ictalurus punctatus*) and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) infected with *Edwardsiella ictaluri*. Peptide treatment groups include cathelicidin-alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with saline only (iCtrl) while the non-infected controls (nCtrl) received no bacteria. The values represent mean ± SEM of three fish and analyzed by one-way ANOVA followed by Tukey's test. *($P < 0.05$)

3.4. Serum protein, albumin and globulin levels

Total protein increased in cathelicidin-alligator and sea snake peptide treatments for hybrid catfish ($P < 0.05$) (Figure 12B). Although both cathelicidins have higher total protein levels, the difference between other peptides and controls were not significant in channel catfish (Figure 12A). The same trend was observed for albumin and globulin levels of both channel catfish and hybrid catfish genetic types (Figure 12). Though albumin and globulin levels increased in the peptide treatment groups, the values were not significant from controls ($P > 0.05$).

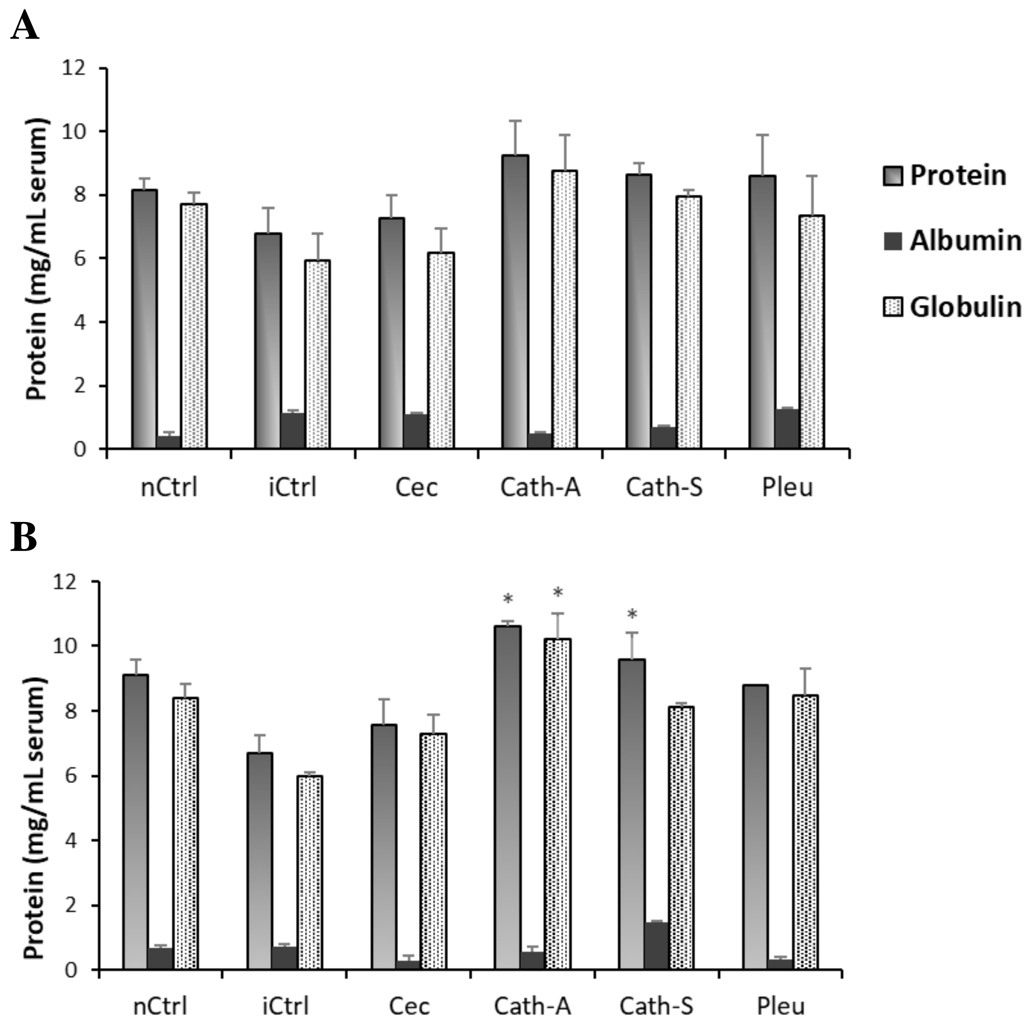

Figure 12 Effect of different antimicrobial peptides on serum total protein, albumin and globulin levels of (**A**) channel catfish (*Ictalurus punctatus*) and (**B**) hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂) infected with *Edwardsiella ictaluri.* Peptide treatment groups include cathelicidin-alligator (Cath-A), cathelicidin-sea snake (Cath-S), cecropin B (Cec) and pleurocidin amide (Pleu). The infected control groups were injected with saline only (iCtrl) while the non-infected controls (nCtrl) received no bacteria. The values represent mean ± SEM of three fish and analyzed by one-way ANOVA followed by Tukey's test. *($P < 0.05$).

4. Discussion

Just like other antimicrobial peptides, most cathelicidins display broad-spectrum antimicrobial activity and have many potential roles in innate immunity. Cathelicidins have been described mostly in vertebrates (Hancock and Sahl, 2006; Kosciuczuk et al., 2012) but limited in fish (Uzzell et al., 2003; Chang et al., 2006; Lu et al., 2011). Recently, cathelicidins were found and characterized in alligator (Barksdale et al., 2017) and sea snake (Wei et al., 2015), in which, aside from direct antimicrobial properties, other functions of cathelicidin such as immune modulation, anti-inflammatory, wound healing, pro-angiogenic activity, and others were elucidated. Considering the *in vitro* efficacy of cathelicidins in previous studies, can they enhance the resistance of channel catfish and channel-blue hybrid catfish from bacterial infections particularly from *E. ictaluri*?

Mortality following challenge with *E. ictaluri* was rapid. The challenge was terminated on day 8 after cumulative total mortality reached approximately 80 % (data not shown). The infected controls began dying early at day 3 and mortality progressed to 90 % at day 8. Previous challenges with *E. ictaluri* in channel catfish done in our lab (unpublished results) and with other studies (Plumb and Shoemaker, 1995; Lim et al., 2009) lasted for more than 20 days. The rapid onset of mortality in this particular challenge might be attributed to several factors such as size and age of fish (mean weight 10g and 60 days of age), species of fish, host's innate resistance, strain of pathogen, mode of infection and prior exposure. Another important factor to consider is the use of an iron chelator incorporated during the culture of *E. ictaluri* which may have enhanced the virulence of the pathogen. Iron is essential for almost all living organisms and lack of iron in bacteria can cause stress which can lead to changes in virulence gene expression (Massé and Arguin, 2005). During challenge, *E. ictaluri* encounters iron starvation stress in the

gastric environment of catfish during the initial phase of infection. The host tends to chelate the free iron using high affinity proteins as a defense mechanism, which in turn, limits iron availability for bacteria (Payne, 1993; Ratledge and Dover, 2000). This mechanism results in low levels of iron in the environment and often trigger virulence factor expression in many pathogens (Litwin and Calderwood, 1993). In *E. ictaluri*, potential virulence genes have been identified during iron restriction; namely the *fur* gene (Santander et al., 2012), *tonB* gene (Abdelhamed et al., 2017) and several proteins including EsrB, LamB, MalM, MalE and FdaA (Dumpala et al., 2015).

The resistance of channel catfish and hybrid catfish to *E. ictaluri* infection was enhanced by cathelicidins derived from alligator and sea snake. Survival rate in channel catfish was highest in fish injected with cathelicidin-sea snake, while in hybrid catfish, both cathelicidin-alligator and cecropin had high survival rates. Variations on the type of cathelicidin and ESC resistance between channel catfish and hybrid catfish may be attributed to host's innate resistance and complex factors including activation of receptors at the time of pathogen exposure. Nevertheless, hybrid catfish had higher mean survival rate of 34 % than channel catfish (28 %) in this study ($p = 0.550$). These results are in agreement with previous studies that channel-blue hybrids had higher resistance to *E. ictaluri* infection than channel catfish following bath immersion in experimental aquaria (Wolters et al., 1996) and in a natural epizootic in pond (Dunham et al., 2008). Furthermore, both cathelicidins significantly decreased bacterial numbers in the kidney and liver of channel catfish and hybrid catfish after 24 h post-infection. This suggests that cathelicidins could endow protection in catfish by eliminating the invading pathogen.

Immune-related responses such as serum bactericidal activity, lysozyme and serum proteins were evaluated following *E. ictaluri* infection. Significant reduction in bacterial colonies were observed in channel catfish and hybrid catfish injected with cathelicidins from alligator and sea snake. This connotes an increase in serum bactericidal activity which may be attributed to elevated levels of various humoral factors in the serum to protect the host from bacterial infection (Rao et al., 2006). Moreover, enhanced lysozyme activity and increased serum proteins induced by cathelicidins might contribute to the observed increase in serum bactericidal activity. It can be noted that lysozyme has hydrolase, which can kill bacteria by cleaving bonds in their cell wall and makes them susceptible to osmotic pressure (Imoto and Yagishita, 1971). While increase in serum proteins are good indicators of health status of the fish (Rao et al., 2006), it also measures two major groups of protein in the blood, albumin and globulin. Albumin is important for tissue growth and healing, while globulin is part of the immune system, as immunoglobulins recognize and bind to a particular antigen like those found in bacteria, and aid in their destruction (Magnadottir, 2006).

Two possible explanations can be deduced regarding the efficacy of cathelicidins from alligator and sea snake in protecting channel catfish and hybrid catfish from *E. ictaluri* infection. First, cathelicidins possess two characteristics as part of their bactericidal mechanism, amphipathic molecular structure and a net positive charge. These structures can allow them to easily bind to the negatively charged bacterial membrane, alter the membrane's orientation and insert into the bilayer membrane. This can induce bacterial membrane permeabilization and cell death (Mansour et al., 2014). Additionally, a highly positive charge can contribute to its potent antimicrobial activity as it can allow the peptide molecules to be oriented in the interface region of negatively charged bacterial membranes and decreasing transmembrane penetration into

eukaryotic membranes (Jiang et al., 2008). The net charge of cathelicidin-alligator is +13 (Barksdale et al., 2017), while cathelicidin-sea snake has +12 (Wei et al., 2015), cecropin has +5 (Jan et al., 2010) and pleurocidin has +2 (Cole et al., 1997). This can partially explain the potency of cathelicidins in inhibiting bacterial growth as compared to other peptides used in this study. Several studies also confirmed that an increase in net charge further improved the antimicrobial activity of the peptides (Giangaspero et al., 2001; Jiang et al., 2008; Wei et al., 2015).

Another possible explanation would be the localization of cathelicidins in neutrophils and mucosal surfaces that can contribute to systemic and local defense (Gennaro and Zanetti, 2000). Previous researches have shown that *E. ictaluri* is able to survive and replicate inside catfish neutrophils and macrophages (Shotts et al., 1986; Baldwin and Newton, 1993; Stanley et al., 1994). We can speculate that since the bioavailability of the peptide is fast upon IP injection, cathelicidins may recruit immune cells such as neutrophils, monocytes and/or macrophages to the sites of infection and can phagocytose the invading bacteria. Further study can verify this by measuring the increase in immune cell numbers following IP injection of the peptides and subsequent bacterial challenge. However, Chen et al. (2017) reported that the cathelicidins derived from Chinese alligator, *Alligator sinensis* (As-CATH4~6), were unstable in the plasma, which may mean that they can easily degrade after the functional performance in the host organism. This characteristic can be a promising therapeutic potential for microbial infections since it can avoid the induction of drug resistance and rapid degradation of the peptide ensures that pathogens can be eliminated efficiently by the immune system (Chen et al., 2017).

5. Conclusion

As one of the most prevalent disease affecting farm-raised catfish in the United States, *E. ictaluri* outbreaks pose a serious threat to the industry. Improved approaches to control the disease are needed. This study demonstrated that two cathelicidins derived from alligator and sea snake are potent antimicrobial agents with strong *in vivo* activity against *E. ictaluri* in channel catfish and hybrid catfish. They enhanced the survival rates of channel catfish and hybrid catfish, reduced the bacterial numbers, increased the serum bactericidal activity, activated the lysozyme and increased the serum proteins. Their amphipathic structure and highly positive charge allow them to induce bacterial membrane permeabilization and disruption leading to cell death. Thus, there is a promising potential for overexpressing these peptide genes in transgenic fish to develop disease-resistant catfish lines.

6. References

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CHAPTER FOUR

Insertion of the Alligator Cathelicidin Gene into a Non-Coding Region of the Channel Catfish Genome using a CRISPR/Cas9 Knock-in System

Abstract

CRISPR/Cas9-based gene knockout in animal cells, particularly in teleosts has proven to be very efficient with regards to mutation rates, but precise insertion of exogenous DNA or gene knock-in via the homology directed repair pathway has seldom been achieved. Two types of donor vectors, dsDNA and plasmid DNA, were designed, which were driven by two different promoters, zebrafish ubiquitin promoter and common carp β-actin promoter, harboring a 250-bp homologous sequences flanking both sides of the genomic target locus in a non-coding region of channel catfish (*Ictalurus punctatus*) chromosome 1. We succeeded in integrating with high efficiency an exogenous cathelicidin gene into the targeted region in chromosome 1 using dsDNA and plasmid DNA construct driven by zebrafish ubiquitin promoter. However, upon analysis, integration rates were higher in dead fry than in live fingerlings, indicating either off-target effects or pleiotropic effects. Additionally, we may be targeting a sensitive area of the genome. Furthermore, low levels of mosaicism were detected in the tissues of P1 individuals harboring the transgene, and high transgene expression was observed in the blood of some P1 fish. This can be an indication of localization of cathelicidin in neutrophils and macrophage granules as also observed in most antimicrobial peptides. These findings may contribute to the generation of a more efficient system for precise gene integration in catfish and other aquaculture species, and the development of transgenic, disease-resistant fish.

1. Introduction

Channel catfish (*Ictalurus punctatus*) is an important food fish in the United States (US) and one of the best studied model species for teleost immunity. Efforts have been geared towards increasing disease resistance in this species as 40% of catfish production was lost to disease at the beginning of the current decade (Tucker, 2012). This is the case for aquaculture in general (Owens, 2012). Improved catfish production systems have been adopted and hybrid catfish (*I. punctatus* ♀ x blue catfish, *I. furcatus* ♂), which is more resistant to diseases than channel catfish, are predominantly cultured. The use of hybrids has resulted in increased farm productivity, but to date, there have been few advancements in disease control (Kumar et al., 2019). In 2016, overall direct economic loss due to diseases in the two largest catfish producing states in the US, Alabama and Mississippi, was valued at approximately $32 million (Peterman and Posadas, 2019). One alternative strategy would be to develop disease-resistant fish lines through transgenesis. However, in the past, there has been some inefficiency in the generation of transgenic lines, and targeted gene insertion was almost impossible.

Recently, a new gene editing system known as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology has been applied in some fish species to produce transgenics (Zhong et al., 2016; Khalil et al., 2017; Kishimoto et al., 2018). In this system, the co-delivery of endonuclease Cas9 combined with a synthetic small guide RNA (sgRNA) targeting certain gene(s) into eukaryotic cells can edit the genome by stimulating a double-strand break (DSB) at a desired site(s) (Doudna and Charpentier, 2014). Genome editing using CRISPR/Cas9 has become popular because it is precise, efficient and inexpensive. It has been used in modifying various genes in model fishes such as medaka and zebrafish (Ansai and Kinoshita, 2014; Auer and Del Bene, 2014). In the last five years, it has

been successfully performed in several aquaculture species, including Atlantic salmon (Wargelius et al., 2016), Nile tilapia (Li et al. 2019), common carp (Zhong et al., 2016), channel catfish (Khalil et al., 2017; Elaswad et al., 2018a), sea bream (Kishimoto et al., 2018) and rainbow trout (Cleveland et al., 2018) to generate a variety of phenotypes related to reproduction, fertility, muscle growth and disease resistance.

Eukaryotic genomes include regions comprised of protein coding genes and noncoding DNA (Amaral et al., 2008). Scientists once thought that noncoding DNA was “junk,” with no known purpose. However, recent studies have indicated that noncoding regions are integral to the function of cells, particularly the control of gene activity (Maumus and Quesneville, 2014; Paul and Dean, 2014). For instance, it is now known that channel catfish genome has 100 % function (Liu et al., 2016). Every base has a function, and noncoding sequences act as regulatory and structural elements such as repetitive DNA sequences (Yuan et al, 2018). Other regions of noncoding DNA provide instructions for transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs) and long noncoding RNAs (lncRNAs) (Paul and Dean, 2014; Liu et al., 2016).

Protein coding sequences are ready targets for many CRISPR/Cas9 applications, wherein investigators have generated small insertion and deletion (indel) mutations to disrupt the open reading frames of protein coding genes (Cong et al., 2013; Li et al., 2014). On the contrary, mutation of non-coding sequences using CRISPR/Cas9 system is often difficult to achieve, because small indels caused by a single mutation may not result in a detectable loss of function (Li et al., 2019). Targeting non-coding sequences either through loss-of-function or gain-of-function approaches can be advantageous since these regions are proposed to affect the expression of neighboring or distant genes by acting as signaling, guiding, sequestering or

scaffolding molecules (Canver et al., 2017; Goudarzi et al., 2019). Targeting non-coding sequences might also affect multiple genes resulting in off-target effects. Some studies have successfully obtained large genomic deletion using dual guide RNAs (gRNAs) in mammalian cells and animal models such as mouse and zebrafish (Zhao et al., 2017; Xiong et al., 2018). Deletion mutations of up to 900 bp was recently achieved in channel catfish targeting the toll/interleukin 1 receptor domain-containing adapter molecule (TICAM 1) using a single guide RNA (Elaswad et al., 2018). Moreover, Li et al. (2019) reported that the CRISPR/Cas9 system could effectively generate desirable non-coding sequence mutants in Nile tilapia (*Oreochromis niloticus*). To date, there have been no studies of targeted gene insertion in non-coding regions away from genes, miRNA loci, lncRNA and heterochromatin regions. Such CRISPR/Cas9 directed insertions could be attempted in order to try to prevent or decrease the probability of negative pleiotropic or off-target effects.

The generation of knockout models by disrupting the gene sequence has been successfully performed in several fish species using the CRISPR/Cas9 system, but its efficiency in creating models that require the introduction of a foreign gene into the genomic loci has been very poor and/or limited (Kimura et al., 2014; Yang et al., 2014). Generally, an induced DNA DSB is repaired either by non-homologous end-joining (NHEJ) where there is no donor DNA, or by homology directed repair (HDR) which requires a donor DNA (Gaj et al., 2013). The efficiency of NHEJ-mediated mutation is high because NHEJ occurs throughout the cell cycle and is a dominant repair system in vertebrates, while HDR occurs only during the late S and G2 phase (Gaj et al., 2013; Rouet et al., 1994). Therefore, the efficiency of HDR-mediated editing is generally low and remains a major challenge. Successful integration of exogenous genes can be achieved by designing optimal DNA donor templates. This can be done by selecting a plasmid

donor with at least 1–2 kb of total homology. This methos is usually used for creating large sequence changes in the presence of target cleavage (Dickinson et al., 2013; Yang et al., 2013). In contrast, single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) sequences are used for small sequence changes (Song and Stieger, 2017).

Cathelicidins belong to a class of antimicrobial peptides (AMPs) which have been shown to exhibit broad-spectrum antimicrobial activity *in vitro* and *in vivo* (Amer et al., 2010; Dean et al., 2011). Due to their small size, amphipathic structure and cationic character, these peptides can rapidly diffuse and neutralize a broad range of microbes, allowing them to persist at water-lipid interfaces where they can then disturb microbial membrane components (Ganz et al., 1985). Expression of genes encoding peptides with *in vitro* antimicrobial activity can result in enhanced resistance to bacterial pathogens in transgenic fish (Dunham et al., 2002; Chiou et al., 2014). Recently, cathelicidins derived from American alligator (*Alligator mississippiensis*) were shown to have a strong activity against some Gram-negative bacteria as well as in multi-drug resistant bacterial pathogens such as *Acinetobacter baumannii* and *Klebsiella pneumoniae* (Barksdale et al., 2016; Barksdale et al., 2017). Inserting cathelicidin gene in channel catfish using CRISPR/Cas9 system might enhance its resistance to various pathogens. A transgenic fish encoding an antimicrobial peptide can confer immunity in fish since it will be protected by the expressed peptide from early in development. Also, an innately disease-resistant fish would not require specific vaccination for certain pathogens and thus will provide an economical solution to bacterial disease problems (Dunham et al., 2002). To date, CRISPR/Cas9 has been explored as a knock-in system in model fishes such as medaka and zebrafish (Ansai and Kinoshita, 2014; Auer and Del Bene, 2014; Armstrong et al., 2013), and much less so on farmed fish (Chakrapani et al., 2016), especially on genes relating to disease resistance.

Here, we employed the CRISPR/Cas9 system to induce site-specific DSBs in a non-coding region of the channel catfish genome. To understand the effect of donor DNA templates on the integration rate, double-stranded DNA sequences carrying two different promoters and plasmid DNA donors were used. Microinjection and electroporation trangene delivery strategies were compared. Our objective is to produce disease-resistant lines of channel catfish carrying actively expressing cathelicidin genes with positive biological functions that can be inherited by subsequent generations.

2. Materials and Methods

2.1. Ethical statement

Channel catfish were reared at the Fish Genetics Research Unit, School of Fisheries, Aquaculture and Aquatic Sciences at Auburn University, Alabama, USA. The Institutional Animal Care and Use Committee (IACUC) approved all the experiments in this study.

2.2. Design of donor DNA templates for knock-in

Two types of donor DNA constructs were used: linear double-stranded DNA (gBlock design) and plasmid DNA (Figure 13). We choose to use the American alligator cathelicidin gene. The coding sequence for this cathelicidin gene is 111 bp long (Barksdale et al., 2017). Expression of cathelicidin was driven by two different promoters for the double-stranded DNA (dsDNA) construct; namely, the truncated version of the zebrafish (*Danio rerio*) ubiquitin promoter (1.4 kb) previously tested by Mosimann et al. (2011) and the common carp (*Cyprinus carpio*) β-actin promoter (1.6 kb) (Liu et al., 1990). Detailed sequences of the dsDNA driven by zebrafish ubiquitin promoter and carp β-actin promoter are found in Appendices C and D. The zebrafish ubiquitin promoter was used for plasmid DNA construct, pUCIDT Amp (2.7 kb). The

constructs also included left and right homology arms (250 bp each) derived from chromosome 1 of channel catfish (Database ID: NC_030416.1) spanning bases 19,128,968 to 19,129,468. The dsDNA constructs and the plasmid DNA were synthesized by Integrated DNA Technologies (IDT) (Coralville, Iowa, USA).

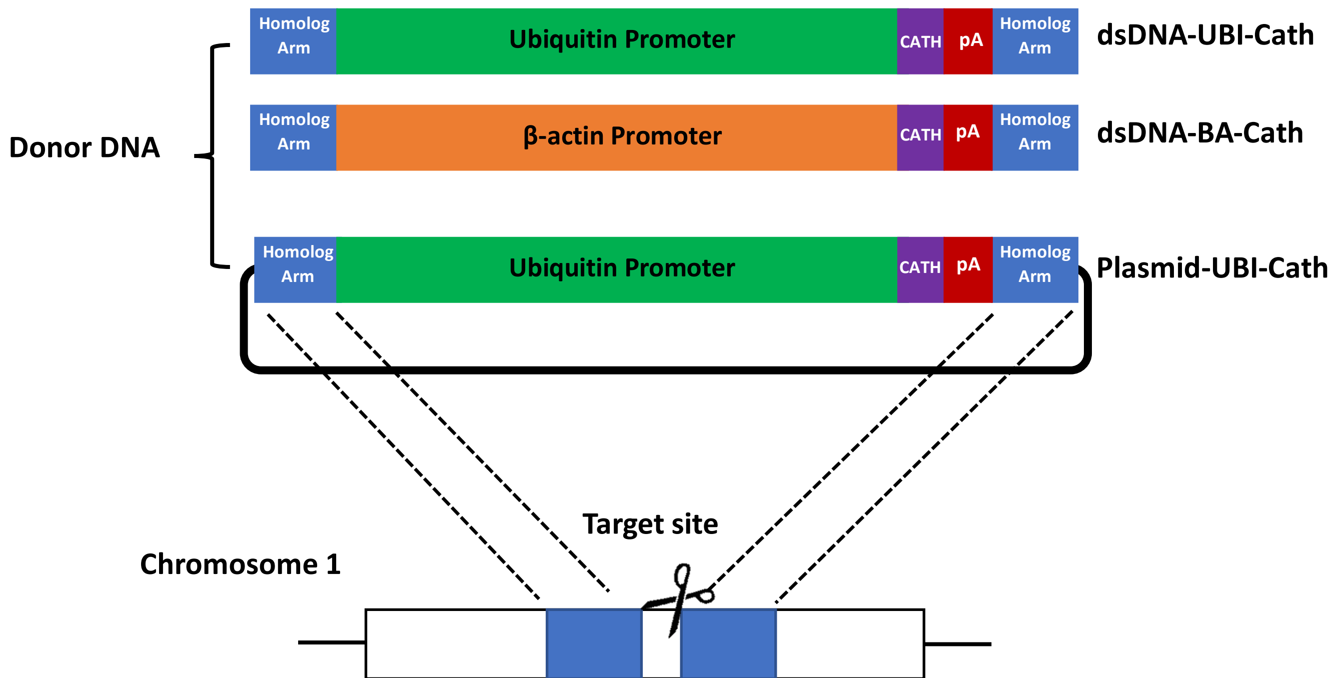

Figure 13 Schematic representation of donor DNA constructs used in CRISPR/Cas9 knock-in of channel catfish (*Ictalurus punctatus*) and the target site in chromosome 1 of channel catfish genome where insertion of the transgene was made. Donor DNA constructs include double-stranded DNA (dsDNA) driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath), dsDNA driven by carp β-actin promoter (dsDNA-BA-Cath) and plasmid DNA, pUCIDT Amp with zebrafish ubiquitin promoter (plasmid-UBI-Cath).

2.3. Design of sgRNA and preparation of CRISPR/Cas9 system

The CRISPR Design online tool (https://zlab.bio/guide-design-resources) was used to design the small guide RNA (sgRNA) that targeted the channel catfish chromosome 1. The protospacer adjacent motif (PAM) sequence (5'-TGG-3') immediately followed the 20 bp target sequence (5'-GTGCTCCTGCTGCTGTTGTA-3'), spanning 19,129,201 to 19,129,221 bp of the chromosome 1 domain. A cloning-free (PCR-based) method was used to generate sgRNA. Table 5 shows the sequences of the universal primer and sgRNA used in this study. The sgRNAs were generated by T7 run –off (Gagnon et al., 2014; Khalil et al., 2017). The universal primer and ssDNA templates were annealed and filled by Platinum™ *Taq* DNA Polymerase (Invitrogen, Waltham, MA). The resulting dsDNA served as the template for *in vitro* transcription to generate sgRNA using the Maxiscript T7 Kit (Thermo Fisher Scientific, Waltham, MA) and was purified using the RNA Clean and Concentrator Kit (Zymo Research, Irvine, CA). The Cas9 protein, which served as the RNA-guided DNA endonuclease enzyme, was obtained from PNA BIO Inc. (Newbury Park, CA). Three different concentrations of the donor DNA for each of the three DNA constructs were prepared: 10, 20, and 40 ng/µL, for a total of nine sets of injection solutions per trial. The CRISPR/Cas9 system used in microinjection was composed of sgRNA, Cas9 protein and donor DNA in the ratio of 1:1:1, including one component of phenol red (60%) to visually track microinjected eggs. The final concentrations of sgRNA and Cas9 protein were 150–200 ng/µL and 300–350 ng/µL, respectively. The sgRNA and Cas9 protein mixtures were incubated in ice for 8 min prior to the addition of donor DNA and phenol red, and then the mixtures were loaded into the microinjection needle (Elaswad et al., 2018b).

Table 5 The sequences of small guide RNA (sgRNA) and the universal (common) primer used to target chromosome 1 (Chr1) of channel catfish (*Ictalurus punctatus*). Underlined sequences represent the protospacer adjacent motif (PAM).

Guide RNA	Oligo sequence (5'-3')
Chr1 sgRNA	GTGCTCCTGCTGCTGTTGTA<u>TGG</u>
Universal primer	TTTTGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACT AGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAAC

2.4. Experimental design

The microinjection experiment had three treatments pertaining to the three DNA constructs designated in this study: dsDNA-UBI-Cath for dsDNA construct driven by zebrafish ubiquitin promoter, dsDNA-BA-Cath for dsDNA construct driven by carp β-actin promoter and plasmid-UBI-Cath for plasmid DNA with zebrafish ubiquitin promoter (Figure 13). Three different concentrations for each of these constructs were used for microinjection namely 10, 20, and 40 ng/µL. There are two control groups: the injected control which contained only phenol red solution (0.5%) diluted in 60 % solution with distilled water (iCTRL) and the non-injected control (nCTRL). For the electroporation experiment, there were three treatments: plasmid microinjection (20 ng/µL plasmid DNA construct), single electroporation and double electroporation. Three control groups were used: injected control (iCTRL, 60% of 0.5% phenol red solution), electroporated control (eCTRL, saline solution only) and non-injected/electroporated control (nCTRL). The microinjection experiment had four replicates while the electroporation experiment had three replicates. The embryos utilized for each replicate were full-siblings, and were subjected to the same handling stress and cultured in the same environmental conditions.

2.5. Egg collection, sperm preparation and fertilization

Broodstock preparation and artificial spawning were performed according to Elaswad et al. (2018b) with modifications. Sexually mature channel catfish males and females were selected for artificial spawning. Female fish were implanted with 100 μg/kg of luteinizing hormone releasing hormone analog (LHRHa) to induce ovulation, and then eggs were stripped in a 20-cm greased spawning pan. On the other hand, males were euthanized, their testes collected, crushed and sperm prepared in 0.9% saline solution. Sperm suspensions (1-2 mL) were added to the eggs and mixed gently. To activate the sperm, sufficient fresh water was added to the eggs to cover the mass; the sperm/egg mixture was gently swirled for 30 s. More fresh water was added and the eggs were allowed to harden for 10–15 min before microinjection.

2.6. Microinjection, electroporation and hatching of embryos

The microinjection solution mentioned above were injected into one-cell stage embryos as described by Khalil et al. (2017) using a microinjection system from Applied Scientific Instrumentation (Eugene, OR). Briefly, 50 nL of the solution were directly injected into the yolk sac of each embryo using a 1.0 mm OD borosilicate glass capillary that was previously pulled into a needle by a vertical needle puller (David Kopf Instruments, Tujunga, CA). Embryos were injected within 15-90 min post-fertilization. The number of embryos injected and their controls for each trial can be found in Appendix A. The injected and control embryos were then reared in 10-L tubs filled with Holtfreter's solution (59 mmol NaCl, 0.67 mmol KCl, 2.4 mmol $NaHCO_3$, 0.76 mmol $CaCl_2$, 1.67 mmol $MgSO_4$) (Armstrong et al., 1989) containing 10 ppm doxycycline. The embryos were incubated with continuous aeration at 27 °C for 6–8 days until hatching. Dead embryos were recorded and removed daily. Those that hatched were transferred to a

Holtfreter's solution without doxycycline until swim up. They were then fed with *Artemia* nauplii three or four times a day once their yolk sac was absorbed. Early fry survival was measured at 15 days post-fertilization and the live fry were reared in 60-L recirculating aquaria systems.

Electroporation of channel catfish sperm and egg were performed as described by Dunham et al. (2018) with modifications. For CRISPR/Cas9 single electroporation, 50 ng/µL of plasmid-DNA-UBI construct, 6 µL of sgRNA and 6 µL of Cas9 were mixed and added into 2 mL of sperm suspension. The mixture was then added to 150-200 eggs in a 10-mL petri dish. Two milliliters of freshwater was then added to activate the sperm. Sperm and egg were incubated for 60 min. For CRISPR/Cas9 double electroporation, sperm suspension containing 25 ng/µL of plasmid-DNA-UBI construct, 3 µL of sgRNA and 3 µL of Cas9 was electroporated first and this was used to fertilize the eggs for 60 min. Then 1 mL saline solution containing 25 ng/µL of plasmid-DNA-UBI construct, 3 µL of sgRNA and 3 µL of Cas9 were added to the eggs and electroporated. Electroporation was carried out using a Baekon 2000 macromolecule transfer system (Baekon, Inc. Saratoga, CA) with parameters set at 6 kV, 2^7 pulses, 0.8 s burst, four cycles, and 160 µs (Powers et al., 1991). Electroporated control embryos contained only Tris-EDTA (TE) buffer. The number of embryos electroporated for each trial and their controls can be found in Appendix B. The same procedure described above was used in rearing the electroporated embryos.

2.7. Integration analysis

Genomic DNA from dead fry and fin-clip samples of 2- to 3-month-old fingerlings was extracted via proteinase K digestion and iso-propanol precipitation as previously described (Kurita et al., 2004). For ease of genotyping a large number of individuals, primer pairs that

could amplify the cathelicidin region for both ubiquitin and β-actin constructs (Table 6) were subjected to PCR amplification. Subsequently, those positive samples were further tested for PCR amplification of 5' and 3' junctional regions in the transgene construct otherwise referred to as promoter and terminal ends, respectively. The same primer sets were used for the dsDNA ubiquitin and plasmid DNA ubiquitin constructs. A positive band indicated a correctly oriented knock-in at the targeted locus. Polymerase Chain Reaction (PCR) products from individual fry were verified by sequencing (Genewiz, South Plainfield, NJ). Integration rates were calculated as the number of positive individuals detected by PCR in a replicate or treatment divided by the total number of individuals in the same replicate or treatment multiplied by 100.

Table 6 Oligonucleotide primers used in determining integration and transgene expression of cathelicidin gene in channel catfish (*Ictalurus punctatus*).

Target gene	Purpose	Name	Nucleotide sequence (5’ → 3’)
Cathelicidin P1 transgene	PCR: Cathelicidin region (Ubiquitin promoter)	Ubi-Cath-F1	GCAGCCAATCACTGCTTGTA
		Ubi-Cath-R1	GTGGTTTGTCCAAACTCATCAA
	PCR: Promoter end (Ubiquitin promoter)	Ubi-PE-F2	GGCTGTTGGTGTAGGGTTTC
		Ubi-PE-R2	GCAGCTAGTGAGTGCTGTGC
	PCR:Terminal end (Ubiquitin promoter)	Ubi-Chr1-F1	GCGGAAAGATCTGGTCATGT
		Ba-Chr1-R2	CAAGTGCAAAGAAGGCAACA
	PCR:Cathelicidin region (β-actin promoter)	Ba-Cath-F1	GACTCCACATGGTCACATGC
		Ba-Cath-R1	GTCTGGATCTCACCGCCTTC
	PCR:Promoter end (β-actin promoter)	Chr1-Ba-F1	CTGTGCTGCTGATGACCATT
		Chr1-Ba-R1	GCGTGCACATTGCTACACTT
		Chr1-Ba-R2	GGCAGATGATATTCCGCACT
	PCR:Terminal end (β-actin promoter)	Ba-Cath-F1	GACTCCACATGGTCACATGC
		Ba-Chr1-R1	TGTTGCCTTCTTTGCACTTG
	qPCR	Ubi-qPCR-F1	TGCTATTCAAGAAGCTGAGGAGG
		Ubi-qPCR-R1	TCATGTCTGGATCTCACCGC
18S rRNA	qPCR	18sF	GAGAAACGGCTACCACATCC
		18sR	GATACGCTCATTCCGATTACAG

2.8. Determination of transgene expression

Expression of the cathelicidin transgene in positive P1 individuals was determined by reverse transcription (RT)-PCR and quantitative real-time PCR (qPCR) analyses. Total RNA was isolated from various tissues using RNeasy Plus Universal Mini Kit (QIAGEN), and for blood samples, RiboPure-Blood Kit (Life Technologies, Carlsbad, CA) was used. One microgram of total RNA was reverse transcribed using iScript™ cDNA Synthesis Kit (Bio-Rad) in a 10-µL reaction volume according to manufacturer's protocol. Then, 1 µL of RT mix was used for the subsequent PCR amplification in a volume of 10 µL containing 3.75 µL nuclease-free water, 0.25 µM of each gene specific primer and 1 µL of EconoTaq® Plus 2x Master Mix (Lucigen, Middleton, WI). The PCR amplification procedure was as follows: initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and 1 min extension at 72°C, and final elongation at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1 % agarose gels.

For qPCR analysis, all cDNA products were diluted to 200 ng/µL and analyzed with a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Gene specific primers were designed using Primer3Plus software. Each amplification was performed in a 10-µL reaction volume containing 5 µL of SsoFast™ EvaGreen® Supermix (Bio-Rad), 1 µL of 5 µM forward and reverse primers, 2 µL nuclease-free water and 1 µL of cDNA. The reaction conditions were as follows: 94°C for 5 s, followed by 40 cycles of 94°C for 5 s, 60°C for 5 s, and a dissociation curve profile of 65-95°C for 5 s/0.5°C increment. Results were expressed relative to the expression levels of 18S rRNA in each sample using the CFX Manager Software version 1.6 (Bio-Rad), and crossing-point (C_T) values were converted to fold differences using the

relative quantification method. Each sample was performed in triplicate. To normalize the starting quantity of RNA, 18S rRNA was used as internal control. PCR using primers Ubi-qPCR-F1 and Ubi-qPCR-R1 should amplify a fragment of 123 bp (Table 6).

2.9. Body weight measurement

Transgenic, non-transgenic, injected control and non-injected control channel catfish were weighed, pit-tagged and transferred to 60-L tanks with 20-30 individuals in each tank approximately 60 days post hatch (dph). This is the first sampling period. The transgenic and non-transgenic fish in each treatment were mixed in the same tank so that they received the same feeding and environmental conditions. The control fish were kept in a separate tank. Fish were fed to satiation 3 x daily (50 % crude protein, Purina AquaMax® Fingerling Starter 300) with the size of pellets appropriate for the body size (stage) of the fish, and were separately adjusted throughout the study period as the animals grew. After another 2 months of growth (120 dph), 36 transgenics, 40 non-transgenics, 35 injected controls and 40 non-injected controls were selected and re-weighed for the second sampling.

2.10. Serum bactericidal assay

Blood was sampled from 15 fish randomly selected from the transgenic group (positive for cathelicidin transgene), the non-transgenic group and the control group (normal channel catfish that did not undergo microinjection). Blood was withdrawn by caudal venipuncture using a 22-gauge needle with syringe. Serum was obtained by allowing the blood to clot overnight at 4°C. Then, serum was collected by centrifugation at 15,000 *x g* for 5 min and stored at -20°C until analyzed.

Bacterial cultures of virulent *Aeromonas hydrophila* strain ML09-119 and *Edwardsiella ictaluri* strain S97-773 were centrifuged and pellets were washed and suspended in 1x phosphate

buffered saline (PBS). Optical density (OD) of the bacterial suspensions was adjusted to 0.2 at 546 nm, then serially diluted (1:10) with PBS five times. To determine serum bactericidal activity, 2 µl of this diluted bacterial suspension was incubated with 20 mL serum in a micro vial for 1 h at 28°C. PBS replaced the serum in the bacteria control group. The number of viable bacteria was determined by counting the colonies grown on nutrient agar plate for 24-48 h at 28°C (Aly et al., 2008).

2.11. Multi-sequence alignment of cathelicidin gene

The cathelicidins used for multi-sequence alignment were obtained from the protein database at the National Center for Biotechnology Information (NCBI). Clustal Omega and Boxshade software were used for the multi-sequence alignment.

2.12. Statistical Analysis

Hatching percentage for embryos was calculated as the total number of fry that have completed hatching divided by the total number of embryos, multiplied by 100. Hatching was completed and recorded after 6- or 7-days post fertilization (dpf). While embryo mortality (%) was calculated as the number of dead embryos in a group or treatment divided by the total number of embryos and multiplied by 100. Fry survival was determined as total number of fry that survived 15 days post hatch (dph) divided by the total number of embryos, multiplied by 100. Integration rates for each treatment were calculated as the total number of positive fish divided by the total number of fish analyzed multiplied by 100. One-way ANOVA and Tukey's multiple comparisons test were used to analyze these data for significant differences among treatments. Histograms were generated in Microsoft Excel 2016 and GraphPad Prism 8 (GraphPad Software, San Diego, CA). Pearson's correlation coefficient was performed to determine the relationship between integration rates and concentration of DNA constructs used

in microinjection. Paired *t*-tests were used to compare integration rates between dead and alive fish positive for transgene. The Shapiro-Wilk test was utilized for analysis of data normality. All statistical analyses were performed using R software (R Core Team, 2014). Statistical significance was set at $P < 0.05$, and all data were presented as the mean ± standard error (SEM).

3. Results

3.1. Embryo hatchability and mortality, fry survival rates

3.1.1. Microinjection

No significant differences were detected in the hatchability of microinjected embryos among the different concentrations of donor DNA for dsDNA-UBI-Cath and dsDNA-BA-Cath (Table 7, Figure 14A). However, for the plasmid-UBI-Cath treatment, low hatch rate was obtained at the 40 ng/μL concentration as compared to 10 and 20 ng/μL concentrations ($P < 0.05$). The nCTRL group had the highest hatch percent (75%) when compared to all other groups ($P < 0.01$). No significant correlation was detected among the different concentrations of donor DNA and embryo hatchability for dsDNA-BA-Cath construct, but a negative correlation was found to be significant ($p = 0.019$) between dosage and hatchability for the plasmid-UBI-Cath construct and dsDNA-UBI-Cath ($p = 0.058$). This means that embryo hatchability decreases as the donor DNA concentration increases for these two constructs.

Mortality of embryos ranged from 25 % in the nCTRL group to 78 % in the 40 ng/μL concentration of plasmid-UBI-Cath construct (Table 7, Figure 14B). Similar mortality patterns were observed among the different donor DNA constructs and iCTRL. No significant differences were detected for embryo mortality among the different concentrations of donor DNA for dsDNA-UBI-Cath and dsDNA-BA-Cath, but for plasmid-UBI-Cath, 40 ng/μL

concentration had high embryo mortality as compared to the two lower concentrations ($P < 0.05$). The nCTRL group had the lowest embryo mortality as compared to all other treatment groups ($P < 0.01$). No significant correlation was found among the different concentrations of donor DNA and embryo mortality for all the constructs.

Significant differences in fry survival rates were detected among the different concentrations of each donor DNA construct ($P < 0.05$) (Table 7, Figure 14C). For dsDNA-UBI-Cath construct, the 40 ng/µL concentration showed the highest fry survival rate (73 %) as compared to two other dosages. In addition, a positive correlation ($p = 0.001$) was also found between dosage and fry survival for the different concentrations of dsDNA-UBI-Cath construct: the higher the dosage of donor DNA used, the higher the fry survival. For dsDNA-BA-Cath construct, high fry survival rate (63 %) was observed in 10 ng/µL concentration. However, no correlation was found between dosage and fry survival ($p = 0.186$). In contrast, the 10 ng/µL concentration of plasmid-UBI-Cath construct gave the lowest fry survival rate (12 %) as compared to other concentrations. However, no correlation was found between dosage and fry survival ($p = 0.163$) for this type of construct. The nCTRL group had the highest fry survival at 78 % as compared to all other treatment groups ($P < 0.01$) and their controls. The iCTRL group had the lowest fry survival as compared to all other treatment groups ($P < 0.01$).

Table 7 The survival and hatchability of embryos, mortality of embryos and fry survival of channel catfish (*Ictalurus punctatus*) microinjected with three transgene constructs in different concentrations carrying the cathelicidin gene utilizing the CRISPR/Cas9 system. Treatment groups are dsDNA construct driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath), dsDNA construct driven by carp -actin promoter (dsDNA-BA-Cath) and plasmid DNA with zebrafish ubiquitin promoter (plasmid-UBI-Cath). Control groups include the injected control (iCTRL, 60% phenol red solution) and non-injected control (nCTRL). Fry survival rate was calculated 15 days post hatch. Hatching was completed and recorded after 6- or 7-days post fertilization (dpf). All data are presented as mean ± standard error (SEM) and analyzed by one-way ANOVA followed by Tukey's test. Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Treatment	Donor DNA (ng/µL)	Number of embryos injected	Live embryos and hatch		Dead embryos		Fry survival	
			N	%	*N*	%	*N*	%
dsDNA-UBI-Cath	10	434	180	41.5 ± 5.11[b]	254	58.5 ± 5.94[b]	73	40.6 ± 6.22[c]
	20	400	137	34.3 ± 3.50[b]	263	65.8 ± 11.72[b]	54	39.4 ± 5.32[c]
	40	386	117	30.3 ± 2.42[b]	269	69.7 ± 13.0[b]	86	73.5 ± 5.51[a]
dsDNA-BA-Cath	10	417	168	40.3 ± 6.41[b]	249	59.7 ± 11.33[b]	106	63.1 ± 8.40[a]
	20	442	139	31.4 ± 5.45[b]	303	68.6 ± 10.71[b]	75	54.0 ± 9.71[b]
	40	388	141	36.3 ± 7.82[b]	247	63.7 ± 13.52[b]	72	51.1 ± 2.70[b]
Plasmid-UBI-Cath	10	372	129	34.7 ± 2.46[b]	243	65.3 ± 13.40[b]	15	11.6 ± 6.73[e]
	20	344	114	33.1 ± 6.32[b]	230	66.9 ± 8.64[b]	55	48.2 ± 7.62[b]
	40	350	77	22.0 ± 10.0[c]	273	78.0 ± 12.80[a]	29	37.7 ± 7.10[c]
iCTRL	-	300	128	42.7 ± 8.65[b]	172	57.3 ± 10.92[b]	35	27.3 ± 4.10[d]
nCTRL	-	300	224	74.7 ± 6.91[a]	76	25.3 ± 9.10[c]	175	78.1 ± 10.0[a]

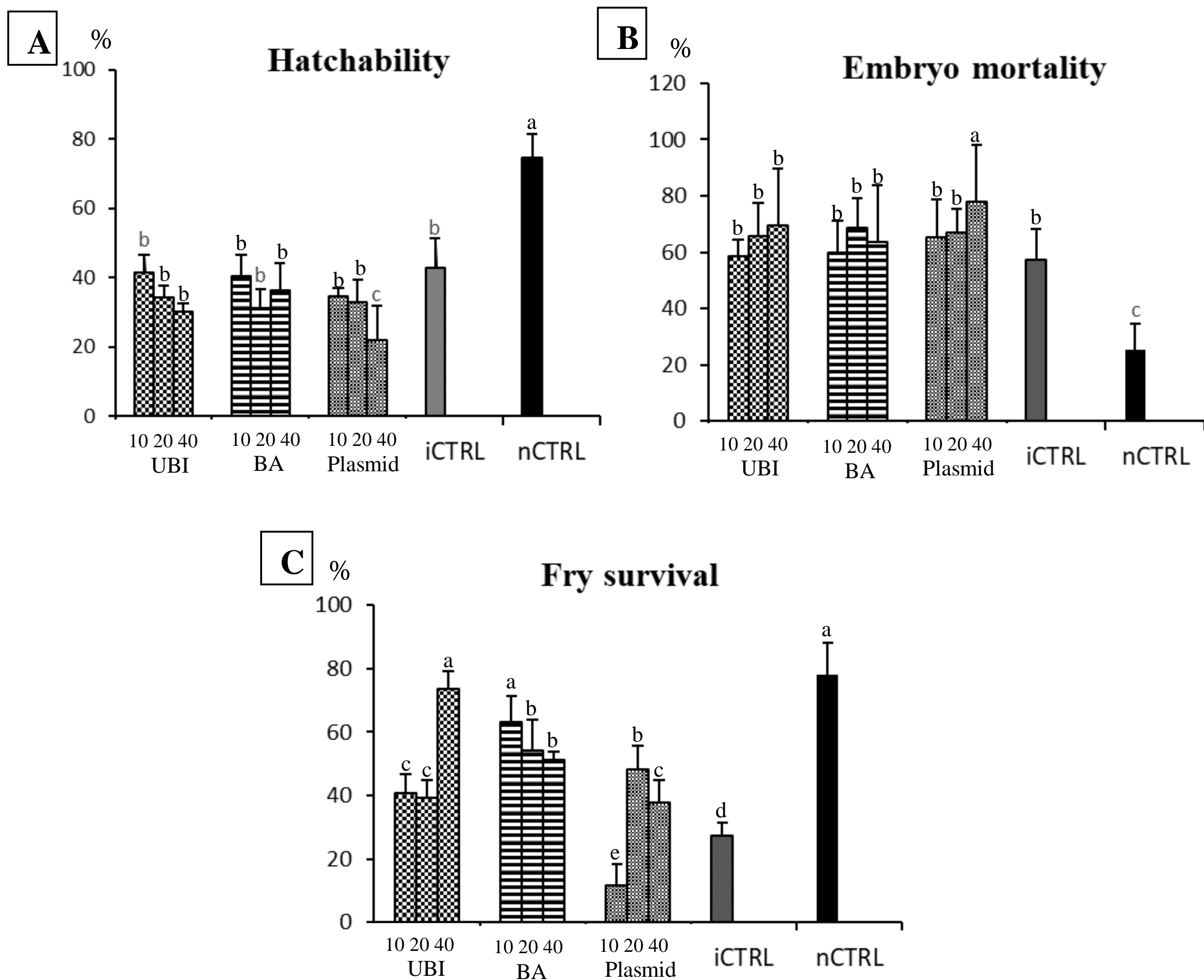

Figure 14 Plots of (**A**) embryo hatchability, (**B**) embryo mortality, and (**C**) fry survival, of channel catfish (*Ictalurus punctatus*) microinjected at one-cell stage with three transgene constructs: UBI (dsDNA driven by zebrafish ubiquitin promoter), BA (dsDNA driven by carp β-actin promoter) and plasmid (plasmid DNA construct with zebrafish ubiquitin promoter) in different concentrations (10, 20 and 40 ng/µL) carrying the cathelicidin gene utilizing the CRISPR/Cas9 system. Control groups included the injected control (iCTRL, 60% phenol red solution) and non-injected control (nCTRL). The values represent mean ± SEM and analyzed by

one-way ANOVA followed by Tukey's test. Means with different letters are significantly different ($P < 0.05$).

3.1.2. Microinjection versus electroporation

To determine the effects of transgene delivery strategies on embryo hatchability and fry survival, microinjection method was compared with electroporation. Hatching rate was highest in eCTRL and nCTRL treatments, while lowest was in microinjection ($P < 0.05$) (Table 8, Figure 15A). Overall comparison of embryo mortality revealed significant differences ($P < 0.05$) (Table 8, Figure 15B). Microinjection (MI) had the highest percentage of dead embryos at 57 %, followed by injected control (iCTRL) at 43 % and single electroporation (SE) at 39 %. The non-injected/electroporated control (nCTRL) had the lowest embryo mortality at 28 % ($P < 0.05$). For fry survival rate (Table 8, Figure 15C), the three control groups, injected control (iCTRL), electroporated control (eCTRL) and non-injected/electroporated control (nCTRL) had high percent fry survival ($P < 0.05$) at 48, 53, and 54 %, respectively as compared to the treatments groups.

Table 8 The survival and hatchability of embryos, mortality of embryos and fry survival of channel catfish (*Ictalurus punctatus*) with different transgene delivery methods. Control groups included the injected control (iCTRL, 60% phenol red solution), electroporated control (eCTRL, saline solution only) and non-injected/electroporated control (nCTRL). Fry survival rate was calculated 15 days post hatch. Hatching was completed and recorded after 6- or 7-days post fertilization (dpf). All data are presented as mean ± standard error (SEM) and analyzed by one-way ANOVA followed by Tukey's test. Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Treatment	Number of embryos	Live embryos and hatch		Dead embryos		Fry survival	
		N	%	*N*	%	*N*	%
Microinjection	280	120	42.8 ± 1.16^{c}	160	57.2 ± 1.17^{a}	77	27.3 ± 1.86^{b}
Single electroporation	300	181	60.9 ± 7.59^{b}	119	39.1 ± 7.59^{a}	80	27.1 ± 4.42^{b}
Double electroporation	288	180	63.5 ± 7.07^{b}	98	33.4 ± 4.26^{b}	85	30.1 ± 4.15^{b}
iCTRL	161	91	56.8 ± 6.12^{b}	70	43.2 ± 6.12^{a}	77	47.8 ± 1.31^{a}
eCTRL	160	112	70.0 ± 0.32^{a}	48	30.0 ± 0.32^{b}	85	52.8 ± 3.56^{a}
nCTRL	160	115	69.7 ± 0.51^{a}	45	28.1 ± 1.20^{b}	86	53.9 ± 1.51^{a}

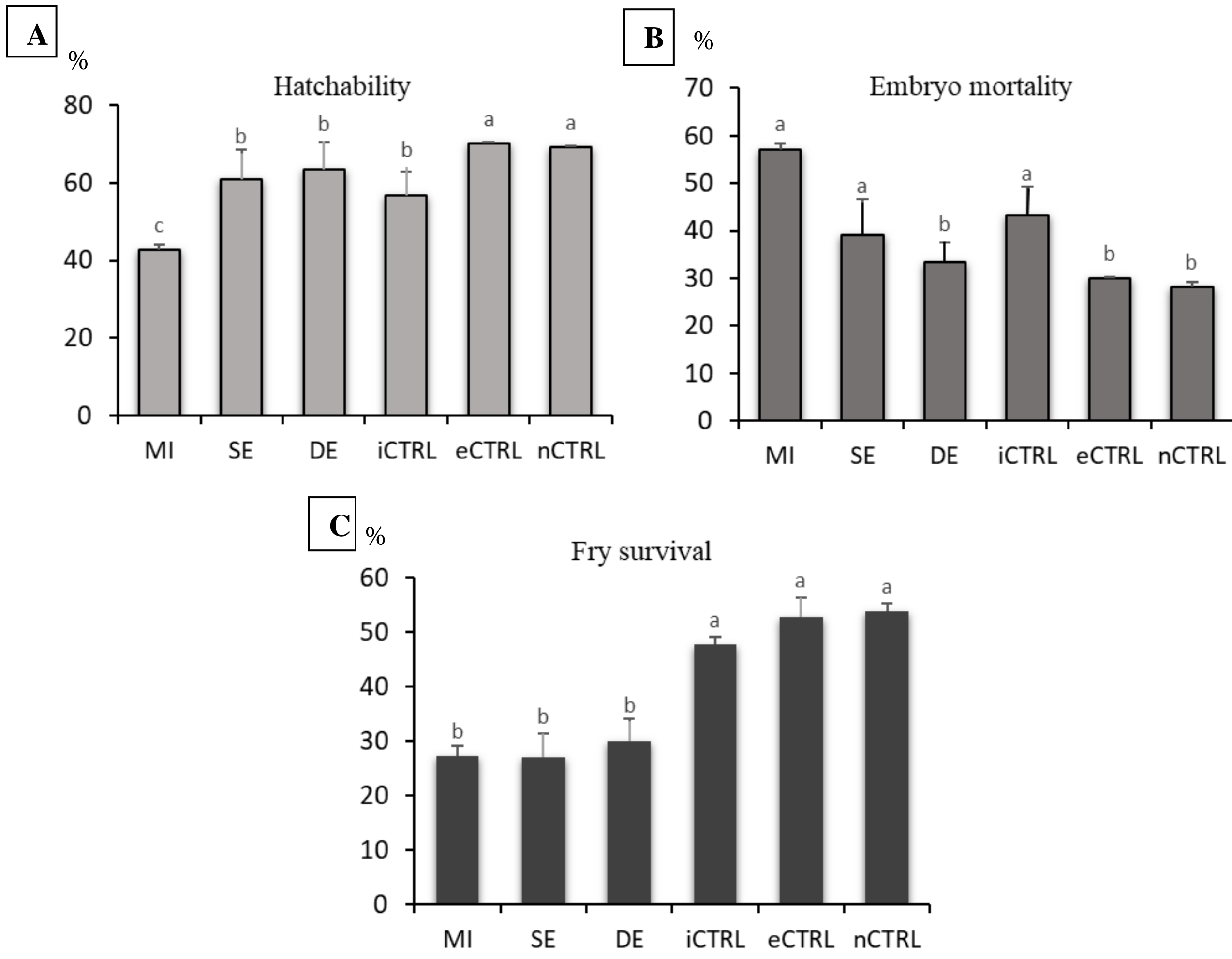

Figure 15 Plots of (**A**) embryo hatchability, (**B**) embryo mortality, and (**C**) fry survival, of channel catfish (*Ictalurus punctatus*) subjected to different transgene delivery methods carrying the cathelicidin gene utilizing the CRISPR/Cas9 system. Treatment groups include microinjection (MI), single electroporation (SE) and double electroporation (DE) using plasmid DNA construct. Control groups included the injected control (iCTRL, 60% phenol red solution), electroporated control (eCTRL, saline solution only) and non-injected/electroporated control (nCTRL). The values represent mean ± SEM and analyzed by one-way ANOVA followed by Tukey's test. Means with different letters are significantly different ($P < 0.05$).

3.2. Analysis of integration

Genotyping strategy had two steps: first, amplification of the cathelicidin region of the donor DNA construct to confirm insertion of the gene, and second, amplification of the 5' and 3' junctional regions (Figures 17A and 18A) to ensure that promoter and terminal regions were also inserted. Figure 16A shows the schematics for amplifying the cathelicidin region (black arrows). Digested PCR products revealed that individuals carrying the cathelicidin gene showed one distinct band (468 bp) comparable to the band seen in the plasmid control (C) (Figure 16B). In contrast, wild type (WT) fish exhibited no band. Sequencing results also confirmed the integration of a 111-bp cathelicidin gene in the positive fish (Figure 16C).

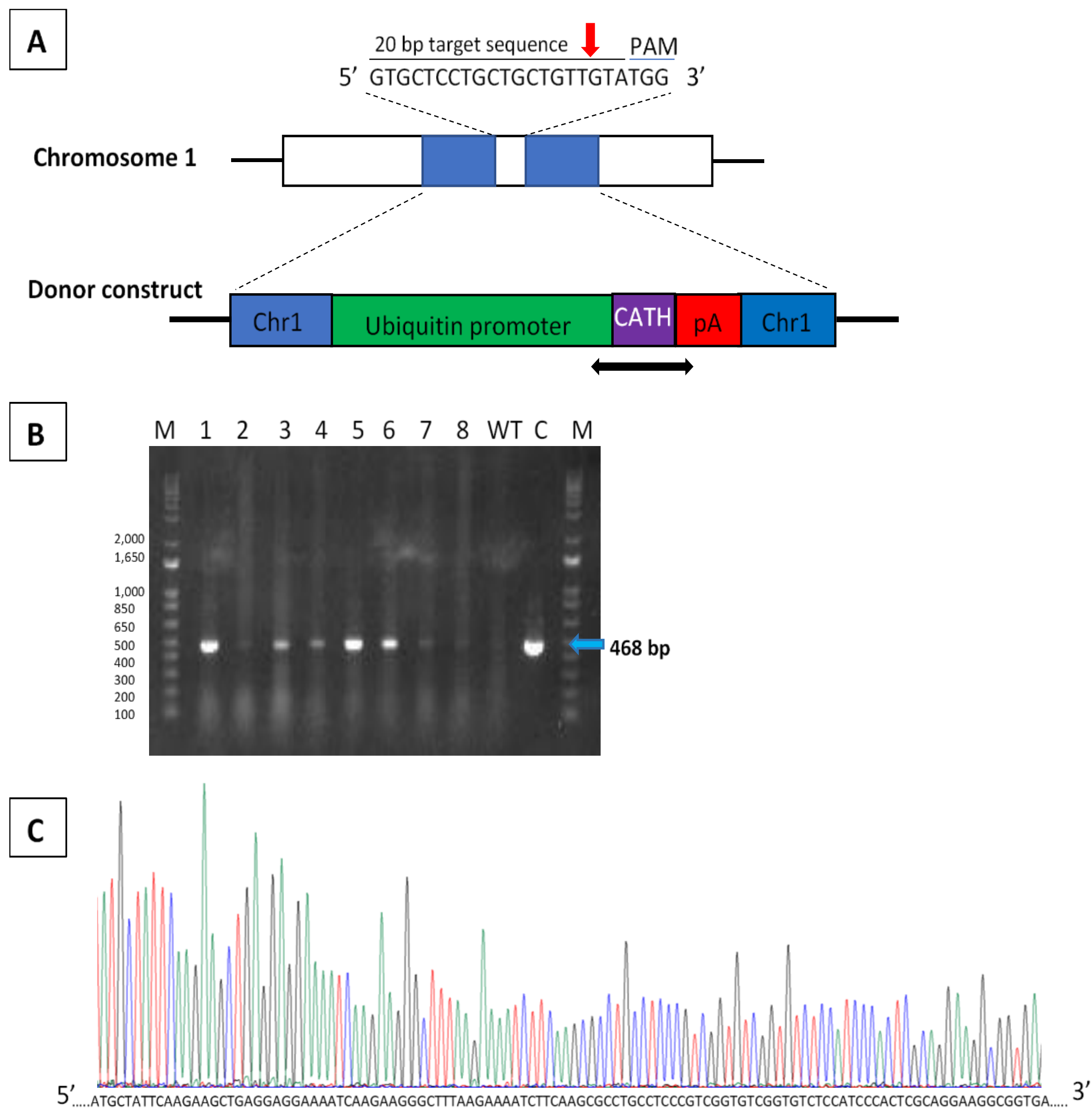

Figure 16 Cathelicidin gene integration at a predetermined site in a non-coding region of channel catfish (*Ictalurus punctatus*) chromosome 1 using CRISPR/Cas9 knock-in system. (**A**) Schematic representation of the 20-bp guide RNA containing the PAM and the cut site (red arrow) (top) which aided the targeted insertion of the donor construct (bottom). Black arrows show primer specific region to amplify cathelicidin gene. (**B**) A representative gel of PCR amplification of cathelicidin gene from positive fish. Lanes 1–8 came from fingerlings

that were microinjected with double-stranded DNA (dsDNA) construct driven by zebrafish ubiquitin promoter with cathelicidin gene, while lane WT represent wild type channel catfish that were full-sib to samples 1–8. Fish positive for transgene have one distinct band at 468 bp. Lane C represents plasmid DNA containing the ubiquitin promoter as positive control and lane M indicates 1 kb plus DNA ladder (Invitrogen). (**C**) A representative sequence chromatogram (Lane 1 of the above PCR-amplified fragment) validated the successful integration of a 111-bp cathelicidin gene in the chromosome 1 of channel catfish genome.

After amplifying the cathelicidin region of the transgene constructs, the 5' and 3' junctional regions of both constructs, dsDNA-UBI-Cath and dsDNA-BA-Cath, were also amplified. Although other primer sets were tried along these junctional and insert-specific regions, three sets of primer pairs worked well for each of the dsDNA transgene constructs. Distinct bands amplified the different regions of the dsDNA-UBI-Cath and dsDNA-BA-Cath transgene constructs (Figures 17A and 18A). The promoter, cathelicidin and terminal regions were amplified by primers sets 1 and 2, 3 and 4, and 3 and 5, respectively. For individuals microinjected/electroporated with plasmid-UBI-Cath construct, the same primer sets were used as in dsDNA-UBI-Cath construct since they have the same promoter. Integration of the cathelicidin and junctional regions were confirmed by sequencing (Figures 17B and 18B). Moreover, the presence of sequences that were not part of the transgene construct (designated by sequences in black, Figures 17B and 18B) but matched the sequences surrounding the homology arms in chromosome 1 of channel catfish further confirmed integration of the whole transgene construct.

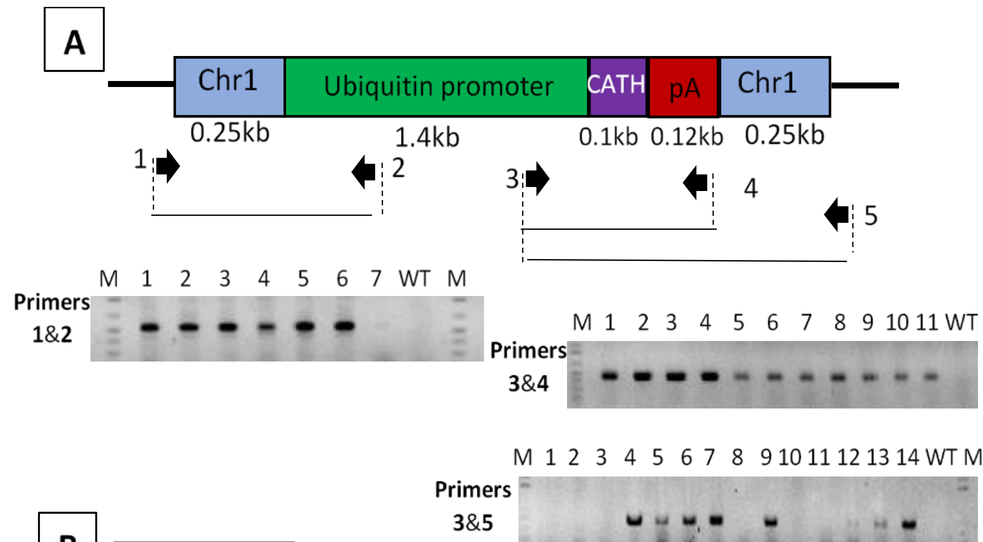

Figure 17 Genotyping strategy for CRISPR/Cas9 knock-in of channel catfish (*Ictalurus punctatus*) using dsDNA construct driven by zebrafish ubiquitin promoter carrying the

cathelicidin gene (dsDNA-UBI-Cath). (**A**) PCR amplification of primer sets at the 5' and 3' junctional regions (primer sets 1&2 and 3&5) and the insert-specific region for cathelicidin gene (primer set 3&4). Numbers in a lane represent individual samples of fish, lane WT represent wild type channel catfish and lane M indicates DNA marker. Presence of a distinct band indicates positive for transgene. Gel electrophoresis images shown here are cropped; full-length gels are presented in Appendix E. (**B**) Representative sequences derived from channel catfish positive for integration of dsDNA-UBI-Cath construct. Sequences in black are the regions outside of homologous arms; Blue sequences are the homologous arms that are part of the transgene construct; Green are partial sequences from the ubiquitin promoter region; Purple are the sequences of cathelicidin gene. Red sequences belong to the poly A terminator sequence. Numbers on the right side of each sequence indicate the number of base pairs in the promoter region, cathelicidin gene region and terminal region as revealed by sequencing of positive fish. Numbers in parentheses are the number of sequencing reactions which yielded positive transgene integration over the total number of sequencing reactions.

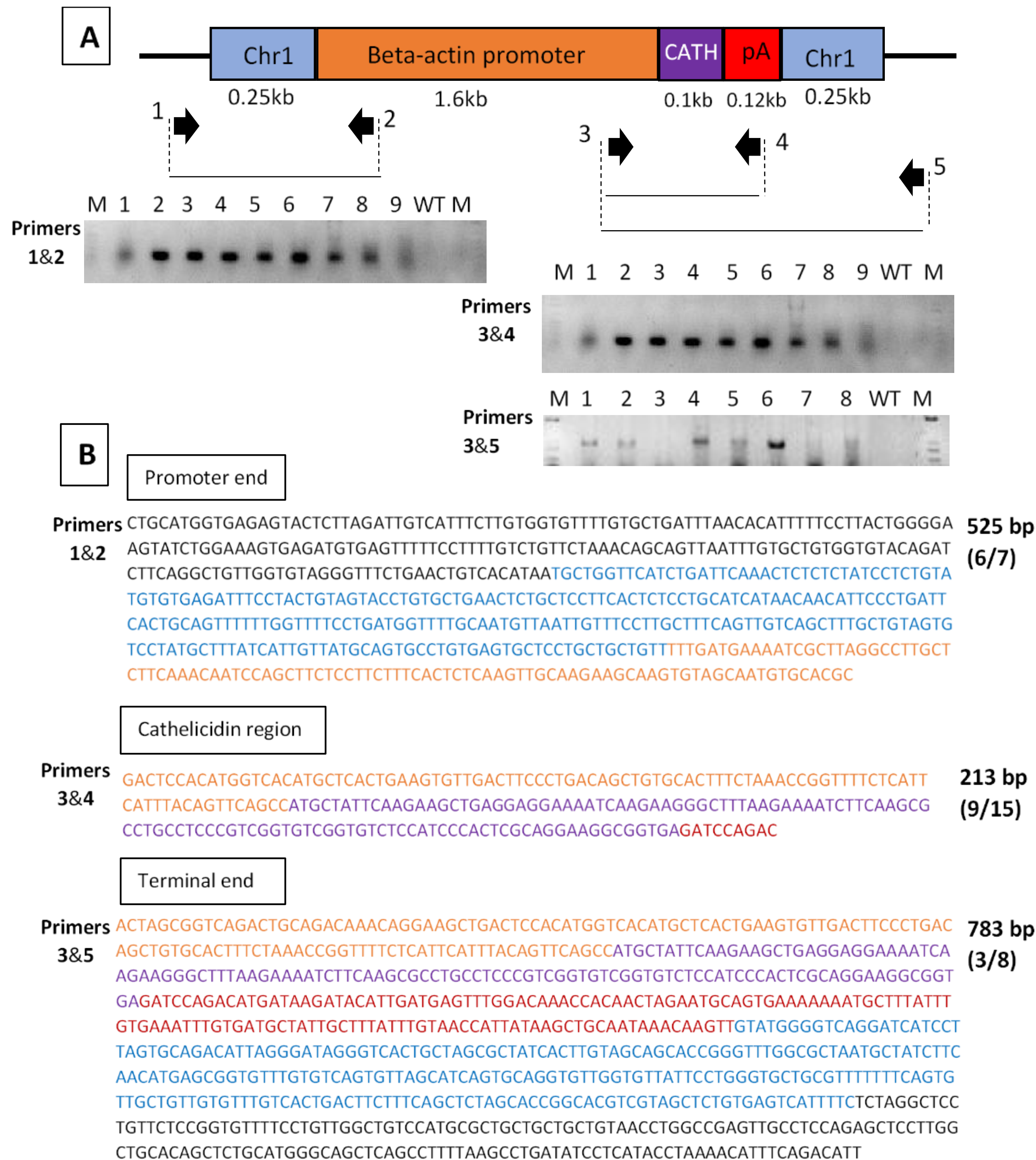

Figure 18 Genotyping strategy for CRISPR/Cas9 knock-in of channel catfish (*Ictalurus punctatus*) using dsDNA construct driven by β-actin promoter carrying the cathelicidin gene (dsDNA-BA-Cath). (**A**) PCR amplification of primer sets at the 5' and 3' junctional regions (primer sets 1&2 and 3&5) and the insert-specific region for cathelicidin gene (primer set 3&4). Numbers in a lane represent individual samples of fish, lane WT represent wild type channel

catfish and lane M indicates DNA marker. Presence of a distinct band indicates positive for transgene. Gel electrophoresis images shown here are cropped; full-length gels are presented in Appendix F. (**B**) Representative sequences derived from channel catfish positive for integration of dsDNA-BA-Cath construct. Sequences in black are the regions outside of homologous arms; Blue sequences are the homologous arms that are part of the transgene construct; Green are partial sequences from the ubiquitin promoter region; Purple are the sequences of cathelicidin gene. Red sequences belong to the poly A terminator sequence. Numbers on the right side of each sequence indicate the number of base pairs in the promoter region, cathelicidin gene region and terminal region as revealed by sequencing of positive fish. Numbers in parentheses are the number of sequencing reactions which yielded positive transgene integration over the total number of sequencing reactions.

3.2.1. Integration rates

Integration rates for dead fry and fingerlings were calculated and compared for each type of transgene construct with different delivery strategies (Table 9). Overall, high integration rates in dead fry were found for 20 and 40 ng/µL concentrations of plasmid-UBI-Cath construct at 78 % and 64 %, respectively ($P < 0.05$). The 40 ng/µL concentration of dsDNA-BA-Cath construct, single electroporation and double electroporation had the lowest integration rates at 17 %, 17% and 20 %, respectively ($P < 0.05$). No integration was observed for 10 ng/µL concentrations of dsDNA-BA-Cath construct. For fingerlings, highest integration rate was found for the 20 ng/µL concentration of dsDNA-UBI-Cath construct (29 %) ($P < 0.05$), excluding the 100 % integration rate found in 40 ng/µL concentration of plasmid-UBI-Cath construct, which had only two surviving fish with both positive for the transgene. The lowest integration rate was found for the 10 ng/µL concentration of dsDNA-BA-Cath construct (3%) ($P < 0.05$), while no positive fish were found for the 20 and 40 ng/µL concentrations of dsDNA-BA-Cath construct.

Table 9 Integration rates of cathelicidin gene constructs in dead fry and fingerlings of channel catfish (*Ictalurus punctatus*) that were microinjected and electroporated using the CRISPR/Cas9 system. Treatment groups are dsDNA driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath), dsDNA driven by carp β-actin promoter (dsDNA-BA-Cath) and plasmid DNA with zebrafish ubiquitin promoter (plasmid-UBI-Cath), single electroporation and double electroporation. Integration rate was presented as mean ± standard error (SEM) and analyzed by one-way ANOVA followed by Tukey's test. Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Treatment	Donor DNA (ng/μL)	Dead fry			Fingerlings		
		Total analyzed	Positive fish	Integration rate (%)	Total analyzed	Positive fish	Integration rate (%)
dsDNA-UBI-Cath	10	31	18	58.1 ± 4.11^{b}	64	9	14.1 ± 1.62^{c}
	20	27	16	59.3 ± 5.35^{b}	7	2	28.6 ± 0.00^{b}
	40	17	6	35.3 ± 3.41^{c}	75	13	17.3 ± 3.93^{c}
dsDNA-BA-Cath	10	8	0	0.0 ± 0.00^{e}	115	3	2.6 ± 0.00^{d}
	20	26	11	42.3 ± 2.68^{c}	25	0	0.0 ± 0.00^{d}
	40	30	5	16.7 ± 3.56^{d}	27	0	0.0 ± 0.00^{d}
Plasmid-UBI-Cath	10	32	10	31.3 ± 3.72^{c}	46	5	10.9 ± 2.20^{c}
	20	9	7	77.8 ± 3.22^{a}	2	2	100.0 ± 0.00^{a}
	40	11	7	63.6 ± 5.69^{a}	16	3	18.8 ± 3.23^{c}
Single electroporation	50	40	7	17.5 ± 2.82^{d}	17	3	17.6 ± 2.85^{c}
Double electroporation	50	25	5	20.0 ± 3.57^{d}	21	2	9.5 ± 0.00^{c}

Integration rate was higher for dead fry than for fingerlings for three concentrations of dsDNA-UBI-Cath construct (10, 20 and 40 ng/μL, $p = 0.004$, $p = 0.029$ and $p = 0.026$, respectively) (Figure 19A). For the dsDNA-BA-Cath construct, no positive fish were found in alive fish for the 20 and 40 ng/μL concentrations, but integration rates were high in dead fry at these concentrations. For the 10 ng/μL concentration of dsDNA-BA-Cath construct, no positive fish were found in dead fry samples (Figure 19B). Moreover, high integration rate was also

observed in alive fish as compared to dead fry in 20 ng/μL concentration of the plasmid-UBI-Cath construct ($p = 0.020$) (Figure 19C). This is in contrast to the results obtained with two other concentrations for this construct, 10 and 40 ng/μL, wherein the dead fry had higher integration rates than alive fish ($p = 0.019$ and $p = 0.024$ for 10 and 40 ng/μL, respectively). Overall, dead microinjected fish had a higher integration rate than alive microinjected fish when samples were pooled ($p = 0.022$) (Figure 19D). The same was true for electroporated fish except that there was no statistically measurable difference between the live and dead fish ($P > 0.05$).

Pearson's correlation test revealed a negative correlation between dosage and integration rate in dead fry for dsDNA-UBI-Cath and dsDNA-BA-Cath constructs ($p = 0.005$). As dosage is increased for these two constructs, the integration rate decreases. There was an observed positive correlation between dosage and integration rate in dead fry for plasmid-UBI-Cath construct, but it was not significant ($p = 0.167$). For those fish surviving to fingerling, no significant correlation between dosage and integration rate was found for all the transgene constructs.

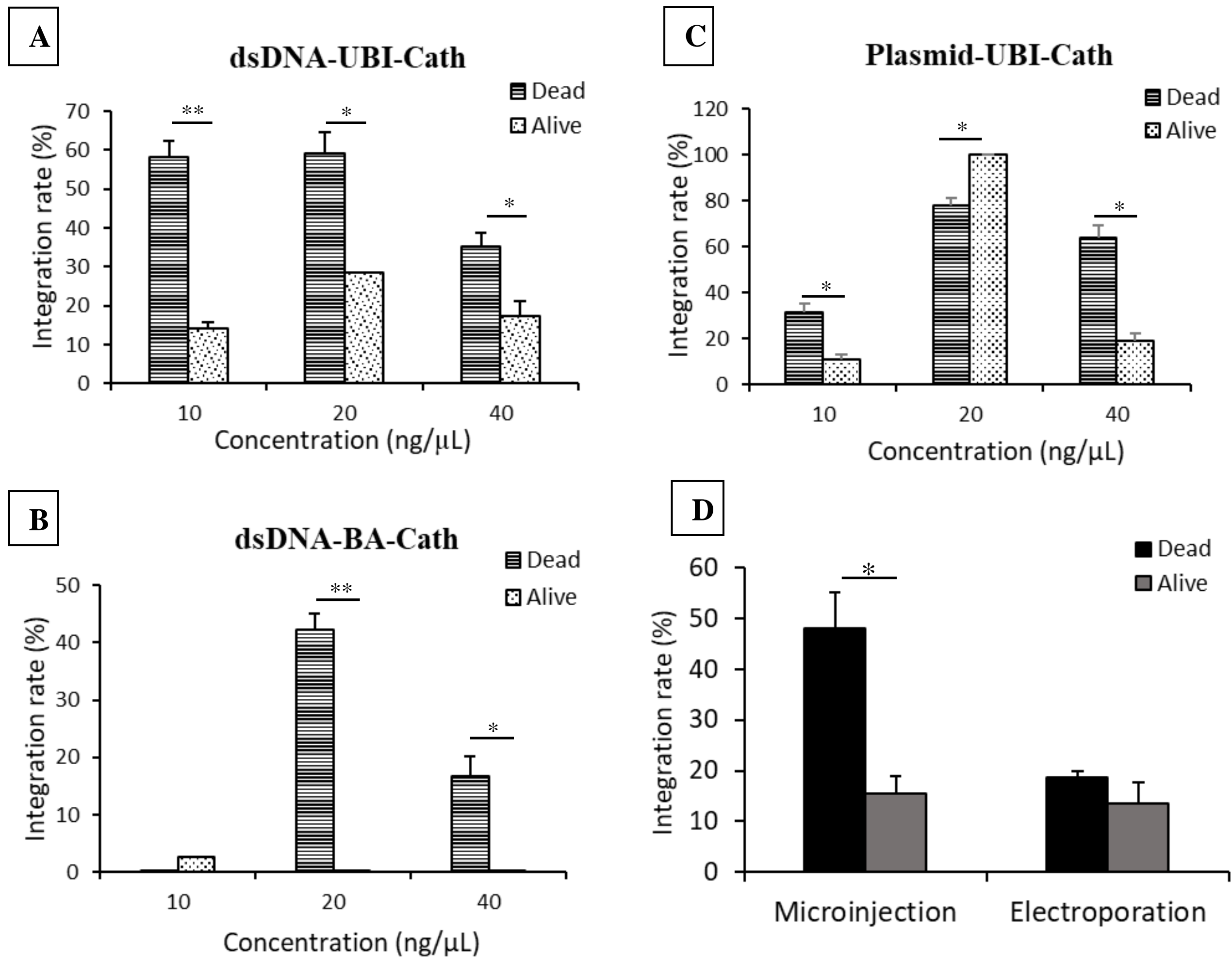

Figure 19 Comparison of integration rates between dead and alive fish of different transgene constructs carrying cathelicidin gene in channel catfish (*Ictalurus punctatus*) using CRISPR/Cas9 knock-in system. (**A**) dsDNA construct driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath). (**B**) dsDNA construct driven by carp β-actin promoter. (**C**) plasmid DNA construct with zebrafish ubiquitin promoter (plasmid-UBI-Cath). (**D**) Data were pooled and integration rates were calculated for microinjection and electroporation. Paired *t*-tests were used to compare integration rates between dead and alive fish positive for transgene. (* $P < 0.05$, ** $P < 0.01$)

3.3. Transgene expression

To detect the expression of cathelicidin transgene in P1 fish, total RNA samples isolated from various tissues of positive fish were subjected to RT-PCR and QPCR analyses. Figure 20A shows that 8 out 10 tissues were found to express the cathelicidin transgene. Strong expression was observed in fin, barbel, eye, muscle, kidney and stomach, while weak expression was observed in heart and intestine. There was no expression detected in gill and liver. To avoid sacrificing potential transgenic fish, blood, fin and barbel were biopsied to isolate RNA. Representative results of cathelicidin transgene expression in two randomly selected positive fish as compared to a non-transgenic fish (analyzed negative for cathelicidin transgene) are shown in Figure 20B. Transgene mRNAs were consistently detected in the blood of both transgenic fish, but the expression in the fin and barbel were varied.

To quantify the level of cathelicidin gene expression in the tissues of transgenic fish, QPCR was performed. High variation was observed among different tissues with a maximum fold change approaching 60 (Figure 21A). Based on the fold changes relative to the liver, cathelicidin mRNA was most abundant in muscle by 57-fold ($P < 0.001$), followed by gill (15-fold), stomach (7-fold) and heart (5-fold). The rest of the tissues have less than 3-fold changes. For the biopsied tissues, three positive fish for cathelicidin gene were sampled for blood, fin and barbel. Based on the fold changes relative to the negative fish (non-transgenic), highest fold change was found in the barbel of transgenic fish 2 (Trans 2) with a 71-fold change and in the blood of transgenic fish 3 (Trans 3), a 53-fold change ($P < 0.001$) (Figure 21B). Relatively high fold change ($P < 0.01$) was also observed in the blood of transgenic fish 1 (Trans 1), while the rest of the tissues have less than 5-fold changes.

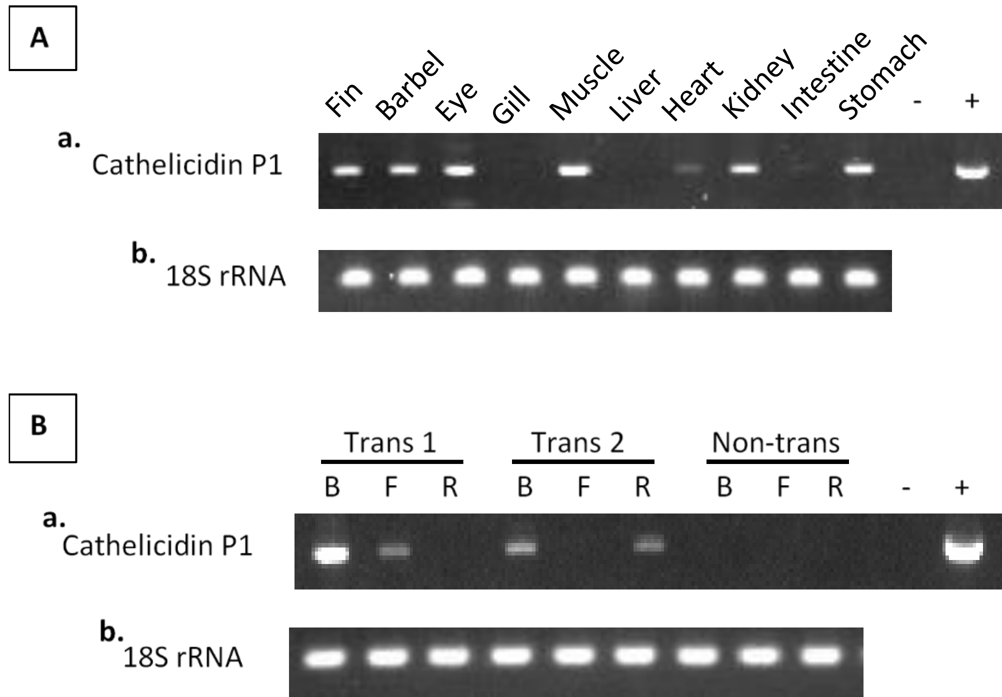

Figure 20 Expression of cathelicidin mRNA in (**A**) different tissues and (**B**) tissues biopsied non-lethally of P1 transgenic channel catfish (*Ictalurus punctatus*), as measured by regular RT-PCR followed by agarose gel electrophoresis. Trans 1 and Trans 2 were P1 transgenic fish and Non-trans was a non-transgenic fish. *B* blood, *F* fin, *R* barbel; +, plasmid DNA positive control; -, PCR reaction in the absence of plasmid DNA. 18S rRNA gene was used as an internal control. Gel electrophoresis images were cropped and full-length gels are presented in Appendix G.

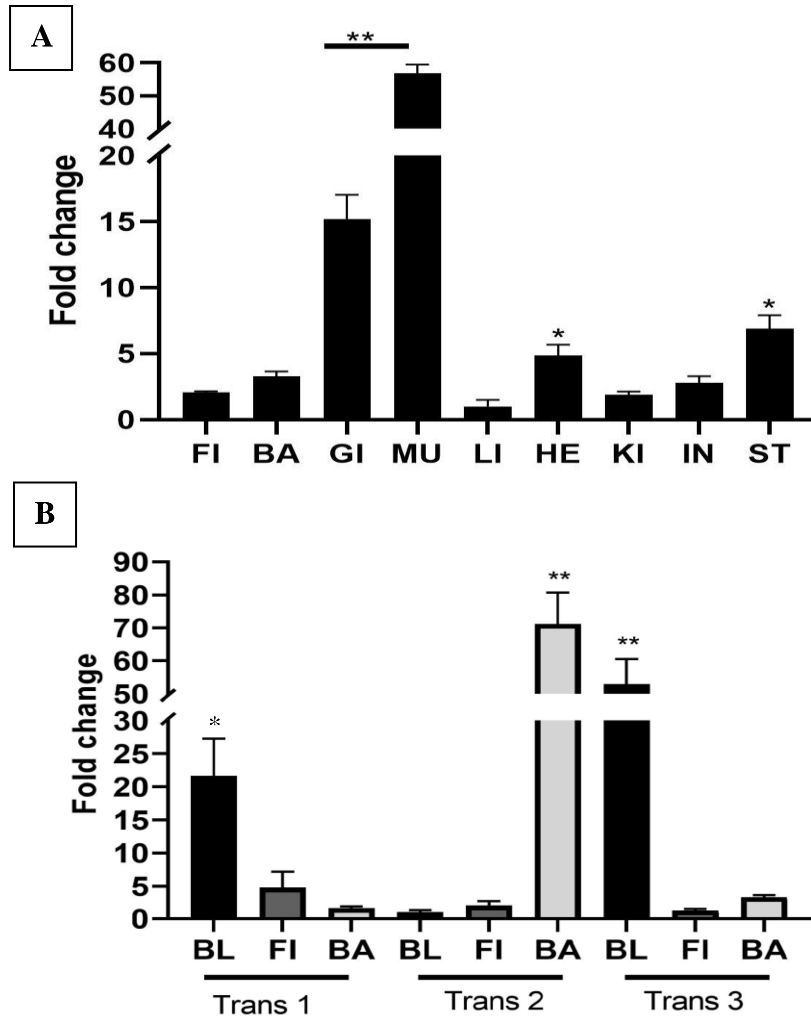

Figure 21 Quantitative analyses of the expression profiles of cathelicidin gene in (**A**) different tissues and (**B**) tissues biopsied non-lethally of P1 transgenic channel catfish (*Ictalurus punctatus*), as measured by QPCR. *FI* fin, *BA* barbel, *GI* gill, *MU* muscle, *LI* liver, *HE* heart, *KI* kidney, *IN* intestine, *ST* stomach, *BL* blood. Trans 1, Trans 2, and Trans 3 were P1 transgenic fish. 18S rRNA gene was used as an internal control for normalization. The expression level was analyzed by $2^{-\Delta\Delta CT}$ method and one-way ANOVA followed by Tukey's test. $^{**}P < 0.001$, $^{*}P < 0.01$.

3.4. Evaluation of body weights

To evaluate whether the expression of cathelicidin P1 transgene in transgenic channel catfish will affect their growth performance when compared to their non-transgenic counterparts and controls, the body weights were measured in two samplings periods: 60- and 120-days post-hatch. Significant differences in the body weights were found among the treatments in the first sampling period (Table 10). Heaviest body weight was observed in the transgenic group, dsDNA-UBI-Cath, and the lowest body weight was found in the same group, but in different treatment (plasmid-UBI-Cath construct) ($P < 0.05$). In the second sampling period, after 60 days additional growth, highest mean weight was observed in the non-injected control (22.6 g) ($P < 0.05$), followed by dsDNA-UBI-Cath construct in the transgenic group (21.2 g), dsDNA-BA-Cath construct in the non-transgenic group (19.9 g) and injected control (16.7 g). The rest of the treatments have lower mean weights than the four treatment groups mentioned above ($P < 0.05$). When data were pooled for the body weights of transgenic and non-transgenic groups in the first and second sampling, no significant differences were observed ($P > 0.05$) (Appendix H). The non-injected control had highest mean body weight in the first and second sampling periods ($P < 0.05$) (Appendix H).

Table 10 Changes in body weights of channel catfish (*Ictalurus punctatus*) microinjected at one-cell stage with three transgene constructs, dsDNA driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath), dsDNA driven by carp β-actin promoter (dsDNA-BA-Cath) and plasmid DNA with zebrafish ubiquitin promoter (plasmid-UBI-Cath). Control groups include the injected control (iCTRL, 60% phenol red solution) and non-injected control (nCTRL). Transgenic fish carries cathelicidin gene inserted into channel catfish genome by CRISPR/Cas9 knock-in system. Transgenic and non-transgenic fish were cultured communally in 60-L tanks with 20-30 individuals per tank, while the controls were kept in separate tanks. Feeding was ad-libitum. Body weight was measured at first sampling (2 months post-injection) and second sampling (4 months post-injection). Data are presented as mean ± standard error (SEM) and analyzed by one-way ANOVA followed by Tukey's test. Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Group	Treatment	*N*	Weight (g)	
			First sampling	Second sampling
Transgenic	dsDNA-UBI-Cath	19	15.1 ± 1.31^{a}	21.2 ± 0.44^{b}
	dsDNA-BA-Cath	3	9.2 ± 0.20^{b}	14.2 ± 1.92^{c}
	Plasmid-UBI-Cath	14	7.7 ± 0.82^{b}	13.4 ± 0.68^{c}
Non-transgenic	dsDNA-UBI-Cath	7	10.1 ± 0.10^{b}	14.2 ± 0.25^{c}
	dsDNA-BA-Cath	25	14.6 ± 0.66^{a}	19.9 ± 0.80^{b}
	Plasmid-UBI-Cath	8	8.1 ± 1.59^{b}	14.6 ± 1.03^{c}
iCTRL	-	35	11.8 ± 0.77^{a}	16.7 ± 0.92^{b}
nCTRL	-	40	14.3 ± 0.78^{a}	22.6 ± 0.86^{a}

3.5. Serum bactericidal activity

To determine if various humoral factors involved in innate and/or adaptive immunities were elevated in the serum, serum bactericidal activity against *A. hydrophila* and *E. ictaluri* was performed. The viable bacterial counts for *A. hydrophila* (Figure 22A) were not significant between transgenic, non-transgenic and control channel catfish ($p = 0.867$). A similar trend was observed in the serum bactericidal activity against *E. ictaluri* (Figure 22B) for which no significant differences were observed between the three treatments ($p = 0.278$), but the transgenic group had a lower mean viable bacterial count (177±11.9 colonies) as compared to non-transgenic (195±12.6 colonies) and control (206±13.4 colonies) groups.

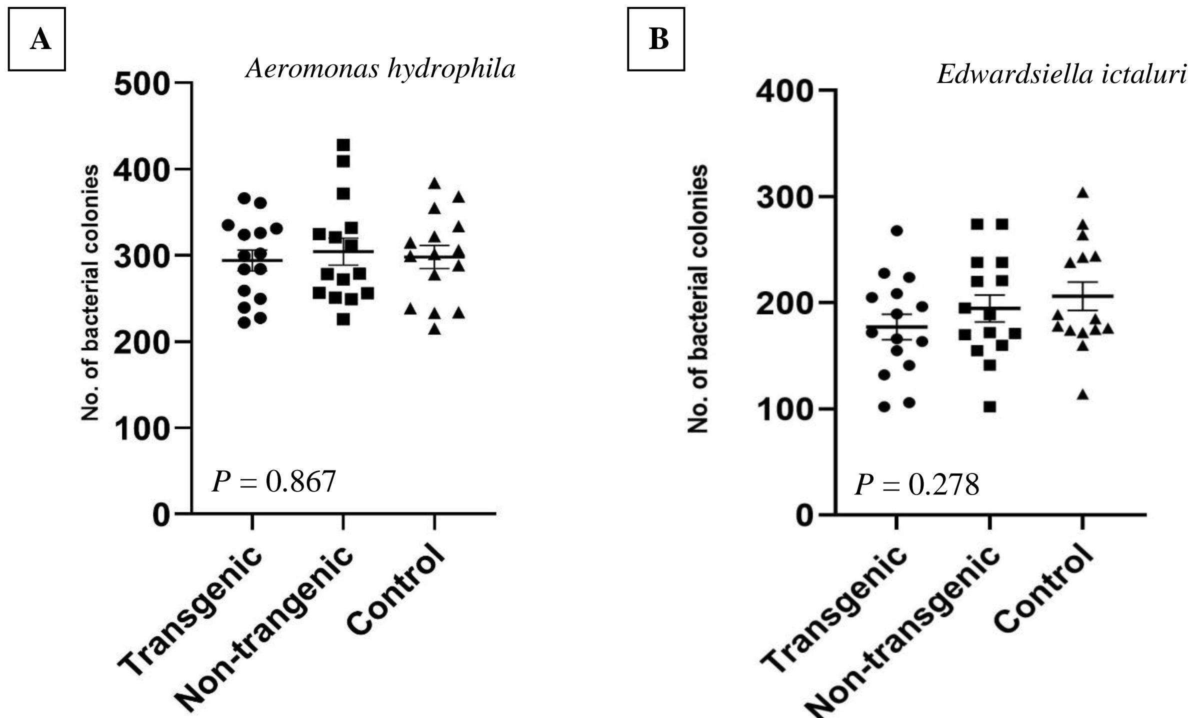

Figure 22 Effect of cathelicidin transgene on serum bactericidal activity of channel catfish (*Ictalurus punctatus*) against (**A**) *Aeromonas hydrophila* and (**B**) *Edwardsiella ictaluri*. Transgenic group includes dsDNA and plasmid DNA constructs carrying cathelicidin gene, non-transgenic group are those found negative for the transgene and control group are wild type channel catfish. Horizontal and vertical bars represent the mean and standard error (SEM), respectively. Data were analyzed by one-way ANOVA. (N = 15 fish per treatment)

3.6. Sequence alignment of cathelicidin-alligator with other vertebrates

Multi-sequence alignment of cathelicidin from American alligator (GeneBank accession number KY044086) with other representative cathelicidins revealed that it has a high degree of similarity with other cathelicidins, especially at the N-terminal region (Figure 23). The four conserved cysteines at the end of cathelin domain of other cathelicidins were also constantly spaced in cathelicidin from American alligator. Cathelicidin from Chinese alligator (Accession number KY828185) had a very similar peptide sequence to the cathelicidin from American alligator. The database (NCBI) was also searched for catfish cathelicidin, but no peptide sequences were found.

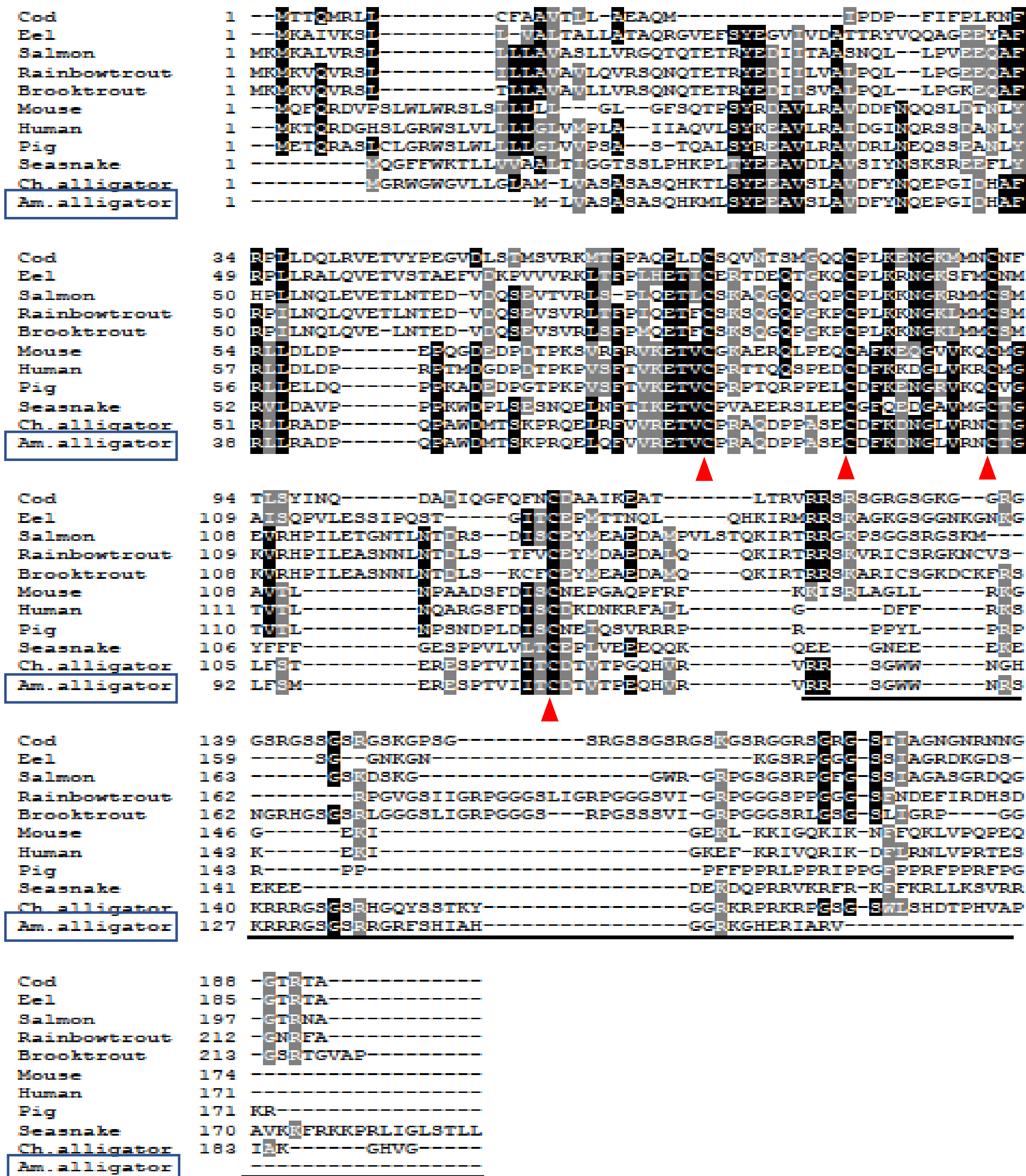

Figure 23 Multi-sequence alignment of different cathelicidins. The alignment was constructed using the Clustal Omega program and edited with Boxshade. Black shading indicates amino acid identity and gray shading indicates similarity (50% threshold). Cathelicidin from American

alligator used in this study is boxed. The four conserved cysteines at the C-terminus of the cathelin domain are marked with red arrows. Mature peptides are underlined. GeneBank accession numbers of the representative cathelicidins are: Human, NP_004336; Pig, NP_999615; Mouse, AAB88303; Sea snake, AKJ54480; Chinese alligator, KY828185; American alligator, KY044086; Atlantic salmon, NP_001117054; Cod, ACE96051, Rainbow trout, AAT67998, Japanese eel, AFP72291; Brook trout, CAQ60110.

4. Discussion

To facilitate a targeted knock-in approach in channel catfish using CRISPR/Cas9 system, a multi-pronged approach was undertaken. Designing an optimal donor DNA template can contribute to increased homology-directed repair (HDR) frequencies (Song and Stieger, 2017), which is favorable for genetic knock-in studies. An effective donor DNA construct was designed to increase integration efficiency, and a non-coding region of the channel catfish genome was edited with CRISPR/Cas9. Then a disease-resistance cathelicidin transgene was inserted into the non-coding region of the channel catfish genome. This has an advantage because ideally, a transgene should not hinder the function of the host genome. Relatively, high integration rates were achieved. This is the first report of targeted gene insertion in the non-coding region away from genes, microRNA loci, long non-coding RNA regions and heterochromatin for an aquaculture species. Several tissues of the transgenic individuals strongly expressed alligator cathelicidin.

The insertion efficiency of dsDNA donor templates in CRISPR/Cas9 system is generally poor (Horii and Hatada, 2016) as compared to single-stranded DNA (ssDNA) donors (Miura et al., 2015; Armstrong et al., 2016; Quadros et al., 2017). Attempts to synthesize a megamer ssDNA donor template in this study failed, due to low GC content of the construct. However, we have successfully generated knock-ins using dsDNA donor templates with integration rate as high as 59 %. These results are comparable to the precise insertion rate of 60 to 90 % using ssDNA donor templates in human cell lines (Sakuma et al., 2016; Aird et al., 2018) and mouse models (Quadros et al., 2017; Gu et al., 2018). Insertion via homologous recombination of dsDNA donor template in mouse models had a knock-in efficiency of ~10% or less (Aida et al., 2015; Wang et al., 2015; Raveux et al. 2017). A knock-in efficiency of 3.5 % was observed in

zebrafish using a dsDNA donor oligonucleotide targeting *C13H9orf72* genomic locus but only 1.7 % showed correct knock-in without additional mutations (Hruscha et al., 2013). In this study, sequence results revealed precise integration of cathelicidin gene without errors (45 out of 45 sequencing reactions) and several sequences contained the complete sequence of the transgene (9 out of 11 sequencing reactions).

Moreover, high integration rates ranging from 31 to 78 % in dead fry and 11 to 100 % in fingerlings were observed with the use of a plasmid donor template. These results were better than the integration rates (via HDR) obtained by the use of plasmid donors in medaka (Murakami et al., 2017) and zebrafish (Irion et al., 2014) embryos, which ranged from 25 to 27 % and 26 to 46 %, respectively. Plasmids as donor vectors for CRISPR/Cas9 transfections are common because they are economical, easy to work with and can include selectable markers. But their drawbacks include random integration of all or part of the plasmid DNA into the host genome, unwanted insertions of plasmid DNA sequences at on-target and off-target sites. Furthermore, plasmid DNA introduced into cells can trigger cGMP-AMP synthase activation, which is stressful to cells (Gabriel et al., 2011; Sun, et al., 2013; Kim et al., 2014). However, sequencing results obtained in this study on samples positive for the transgene with the use of plasmid donor DNA revealed that no unwanted plasmid sequences were integrated into the target site. The optimum donor DNA concentration for the plasmid donor was 20 ng/µL because it gave the highest integration rate in both dead and alive fish. Fortunately, this concentration may be less toxic, since high fry survival rates were also observed at this donor DNA concentration.

The efficiency of HDR generating successful knock-ins in the current study can be due to several factors. First, the design of the homologous donor construct contributed to the efficiency of HDR which led to high integration rates. The length of the homology arms located at each

end of the construct also plays an important role in increasing HDR rate and precise integration. Studies have shown that the efficiency of recombinatorial repair increases as the length of homology arms increases (Li et al., 2014, Song and Stieger, 2017; Zhang et al., 2017). The use of a 250-bp homology arms identical to the sequences surrounding the target site in channel catfish chromosome 1 generated integration rates ranging from 14 to 100 % in live P1 fingerlings. A similar finding was observed by Zhang et al. (2017) in which increased length of homology arms from 50 bp to 300 bp led to targeting the insertion site more effectively in 293 T cells. In that study, Zhang et al. (2017) observed a 77% precise insertion rate of 300 bp homology arms; in contrast, inserts containing 50 bp homology arms had only a 63% insertion rate. Studies that used 1 kb or more homology arms achieved variable results and showed from 12-58 % HDR rate (Byrne et al., 2015; Song and Stieger, 2017). Shorter homology arms would be expected to be more accessible for insertion in the target site during the simultaneous cleavage of genomic and plasmid DNA (Zhang et al., 2017). Also, the donor templates used were designed to have two homology arms identical to sequences surrounding DSB created by Cas9/sgRNA, which resulted in correct insertion of the transgene to the target site. The presence of homologous DNA at the target site can allow HDR to occur so that host genome DNA between homologous arms is exchanged with the donor template, resulting in insertion of the transgene (Zhao et al., 2014).

The design of the sgRNA can also be a factor for achieving precise integration as the sgRNA was designed to cleave at the exact insertion site, which is three nucleotides upstream of PAM (Figure 4A). Previously in our laboratory, this sgRNA worked effectively at the target site to insert desaturase gene in channel catfish chromosome 1 (unpublished results). It has been demonstrated that the farther the guide RNA from the target site, the poorer will be the correct

insertion (Miura et al., 2018). It was hypothesized that DSBs were induced by Cas9 protein at the exact target cut site which led to HDR, precisely inserting the transgene constructs (Song and Stieger, 2017). For circular donor plasmids, the induction of DSBs on sgRNA target sequences next to homologous arms using the CRISPR/Cas9 system has increased targeted integration events (Ochiai, et al., 2012; Irion et al., 2014). This is in contrast to the previously mentioned construct above, on low efficiency of plasmid donors. Circular homologous repair templates can be more efficient than linearized templates since there are no free ends available for binding to the NHEJ protein in circular donors and thus may be favorable for homologous recombination repair to occur (Song et al., 2019). The lack of free ends in circular donors can also protect the cyclic repair template from exonuclease action, thus, extending the time that can be used as a repair template (Nødvig et al., 2018). In addition, the use of only one sgRNA is advantageous because it perfectly synchronized the demand and supply of homologous sequences leading to an efficient cleavage at the cut site. Some studies have used two sgRNAs, one for creating genomic DSB and another for releasing donor template from the plasmid (Hisano et al., 2015; He et al., 2016). However, Zhang et al. (2017) observed that the use of two sgRNAs led to a ~10 % decrease in HDR as compared to the use of one sgRNA, which may be due to different cleavage efficiencies of the two distinct sgRNAs.

One of the key components of an effective transgenesis toolkit is a ubiquitous promoter to drive transgene expression. Each type of promoter has shortcomings in the different developmental stages and cell types of various transgenic organisms. A common setback of most promoter is their progressive inactivation during the developmental stages of an organism (Mosimann et al., 2011). In case of β-actin promoter, some studies have shown that it can maintain transgene expression to adulthood, but sometimes does not show significant activity in

erythrocytes or fins, or several other cell types such as brain, retina, kidney and blood (Traver et al., 2003; Burke et al., 2008). Factors relating to the differential expression pattern utilizing the β-actin promoter may be the potential cell-type-specific requirements controlling translation or chromatin maintenance and differential methylation (Burke et al., 2003; Mosimann et al., 2011). On the other hand, Mosimann et al. (2011) demonstrated that zebrafish ubiquitin promoter could drive strong and ubiquitous expression of an enhanced green fluorescent protein (EGFP) reporter gene in zebrafish and the transgene revealed strong expression in all analyzed external and internal organs, including the retina, fin fold, and across all blood cell types from embryo to adulthood. A truncated version of this promoter was utilized in the design of dsDNA and plasmid DNA construct in the current study, which might explain the high transgene expression observed in the blood of positive fish microinjected with these two constructs.

The different concentrations of dsDNA and plasmid constructs affected the hatchability and mortality of the microinjected embryos. Hatchability of embryos for the dsDNA-UBI-Cath and plasmid-UBI-Cath constructs decreased as dosage of donor DNA was increased. No correlation between hatching rate and dosage was found for the dsDNA-UBI-Cath construct. Although there was no significant correlation, embryo mortality for the three constructs increased as dosage of donor DNA was increased. Elaswad et al. (2018) observed that increasing the gRNA and Cas9 protein concentration during microinjection could lead to decreased embryo hatchability and increased embryo mortality in channel catfish. However, in this study, gRNA and Cas9 protein concentrations were unchanged; only the donor DNA had different concentrations tested, i.e. 10, 20 and 40 ng/µL. The increased embryo mortality could be due to the production of toxic products at higher concentrations of donor DNA which may have adverse effects on the cells. Not surprisingly, higher embryo mortality was observed in

microinjected groups as compared to the non-injected and non-electroporated groups. This phenomenon was also observed by Khalil et al. (2017) and Elaswad et al. (2018) when they microinjected channel catfish embryos at one-cell stage. The main reason for this may be due to the physical stress induced by the microinjection procedure itself to the embryo, which decreased the viability of cells.

Fry in the three constructs had significantly lower survival as compared to the nCTRL indicating the harmful effects of microinjection on early fry survival. The higher the donor DNA concentration for dsDNA-UBI-Cath construct, the higher the fry survival. But this was not the case for the two other constructs where there is no correlation between dosage and fry survival. Moreover, the different transgene delivery methods did not have a significant effect on fry survival. However, the controls (electroporated and microinjected controls) have higher fry survival rates as compared to microinjection and electroporation. Possible causes may be physical stress on the delivery methods to the eggs, low egg quality or some components of sgRNA or Cas9 protein might be toxic to the cells (Shah et al., 2015).

Microinjection remained more effective than electroporation in delivering the transgene construct into the embryos of channel catfish. Although microinjection is laborious, costly and requires skilled personnel, it is a reliable method because it allows precise control over the amount of material introduced into the cell and the timing of introduction (Dunham and Winn, 2014). Overall, high integration rates were obtained in microinjected fish as compared to the electroporated ones. Integration rates were also higher in dead fry than in fingerlings, indicating either off-target effects, pleiotropic effects or lethal overexpression. Additionally, a sensitive area of the genome may have been targeted, although our hypothesis was that this would not be a sensitive area.

Low levels of somatic mosaicism were observed in the tissues of positive founder fish as revealed by RT-PCR and qPCR. For biopsied tissues, transgene expression was consistently expressed in the blood, while variable expression between transgenic fish were found for fin and barbel. A strong donor DNA construct design, an efficient CRISPR/Cas9 system and a genomic target which is away from other genes may have contributed to the decreased mosaicism achieved in this study. Another factor may be due to the timing of introduction of CRISPR/Cas9 components into very early-stage zygotes. CRISPR/Cas9 components along with the donor DNA was injected into one-cell stage embryos within the 90-minute window, just before the beginning of the first cell division. This may suggest that the genome editing process may have occurred before the first genome replication, with the divided cells carrying the exogenous donor DNA. Delivery of CRISPR components earlier in the one-cell stage have reduced mosaicism in mice (Hashimoto et al., 2016), sheep (Vilarino et al., 2017) and bovine (Lamas-Toranzo et al., 2019). In the conventional non-targeted gene transfer, delayed transgene integration during embryonic cell division could result in a mosaic fish (Dunham and Winn, 2014). Also, in non-targeted gene insertion, the insertion site cannot be controlled. This could create random DSBs, thus then the repair process would be inefficient (Lombardo et al., 2011). This may also result in undesired mosaicism. In effect, when the transgene is integrated in only one cell group or tissue but not into germ cells, gene transmission to the offspring would be difficult (Maclean, 1998; Dunham and Winn, 2014). With CRISPR/Cas9 system, programmable nuclease-induced DSB generation at the desired gene insertion site using sgRNA and Cas9 could enhance the efficiency of gene insertion through homology-directed repair (HDR) and could lead to reduced mosaicism (Mehravar et al., 2019).

Evaluation of body weights at 60- and 120-day post-hatch revealed that the fastest growing transgenic fish group were those injected with dsDNA-UBI-Cath construct, which had also the highest integration rate among the alive fish. While the slowest growing transgenic group were those injected with the plasmid-UBI-Cath construct, with the only difference from the dsDNA-UBI-Cath construct was the extra plasmid sequence. The reduced growth seen in fish injected with the plasmid-UBI-Cath construct may be due to overexpression, some toxicity stemming from the plasmid or both, but the exact cause cannot be determined without further study. On the other hand, the dsDNA-BA-Cath construct also generated slow growing fish comparable to the plasmid-UBI-Cath construct. Fry survival for the dsDNA-BA-Cath construct was higher than the other two constructs and generated the greatest number of live fish, but the integration rate in fingerlings was lowest. One possible explanation may be that it was overexpressed or expressed in a sensitive tissue or cell, killing the embryos that integrated this construct. Further validation is needed if there are indeed some pleiotropic effects, especially affecting growth rate with the transgene insertion. The non-injected control group had consistently the highest body weight in the first and second sampling, and when comparing the non-injected control with the injected control, microinjection harmed early growth. Other groups that have lagged growth rate might be affected by the adverse effects of microinjection procedures.

To further evaluate a possible biological function of cathelicidin gene when inserted into the genome of channel catfish, serum bactericidal activity against *A. hydrophila* and *E. ictaluri* were measured *in vitro*. However, no statistically significant differences in the bacterial counts were obtained between transgenic, non-transgenic and control groups. But the transgenic group had observed lower number of bacterial colonies than non-transgenic and control groups.

Statistically significant lower bacterial counts were observed in a previous pathogen challenge tests (unpublished results) when cathelicidin peptide was injected into channel catfish then simultaneously challenged with *E.ictaluri*. Decreased bacterial counts in the pathogen-infected fish may be due to cathelicidin expression being dramatically upregulated in response to a bacterial infection (Kosciuczuk et al., 2012; Cole and Nizet., 2016). When the serum was collected from healthy fish, apparently there was no induction of a pathogen, and thus, low levels of cathelicidin might not be enough to produce a substantial effect on the reduction of bacterial numbers *in vitro*. Cathelicidins are stored as inactive precursors in the secretory granules of neutrophils and macrophages, although low levels can be found in non-myeloid cells such as epithelial cells (Zanetti et al., 1995; Kosciuczuk et al., 2012). They can be released extracellularly as mature peptides when required or upon leukocyte activation (Treffers et al., 2005). Since leukocytes were not activated in the absence of a pathogen, the release of antimicrobial peptides including cathelicidin were also hampered, and perhaps the reason why there was little bactericidal activity *in vitro*.

The localization of cathelicidins in the myeloid tissues might explain why high transgene expression was found in the blood of positive P1 fish (see Figure 21B). Myeloid tissues arise from hematopoietic stem cells in the bone marrow and represent the major leukocytes in the peripheral blood (Kawamoto and Minato, 2004). Cathelicidins are said to be stored in neutrophils and macrophages (Zanetti et al., 1995), which are types of leukocytes and both possess a nucleus which can express genes.

The C-terminal domain which encodes the mature cathelicidin peptide show considerable heterogeneity among species and different peptides, but the cathelin domain and N-terminal region are highly conserved in different cathelicidins (see Figure 23). Recently characterized

cathelicidins from alligator (Barksdale et al., 2017) and sea snake (Wei et al., 2015) both have strong antimicrobial activities against a variety of pathogens. The unique cathelin domain and the conserved N-terminal region among cathelicidins might be another factor which contributed to the efficacy of cathelicidin peptide from alligator to inhibit catfish pathogens in previous challenge tests done in our lab (unpublished results). The next steps would be to unravel its role in disease resistance in channel catfish encoding this peptide. If we observe elevated resistance against catfish pathogens in an F1 generation expressing the transgene, we could confirm the efficacy of the targeted gene insertion reported here.

6. Conclusion

In this study, we demonstrated that targeted gene integration can be performed using a CRISPR/Cas9 knock-in system in channel catfish. Effective design of a donor template incorporating the zebrafish ubiquitin promoter, presence of a 250-bp homologous arms at each end of the construct and a sgRNA designed to cleave at the exact insertion site, all contributed to increased HDR rate, leading to precise integration of the transgene construct into chromosome 1 of channel catfish genome. Evaluation of the P1 individuals revealed low levels of somatic mosaicism. This can be due precise genome editing by CRISPR/Cas9 and timing of introduction of Cas9 into one-cell stage embryos. We interpret the success of this work to mean that genome editing processes may have occurred before the first chromosomal replication. The highest integration rate occurred among live founder fish treated with the dsDNA-UBI-Cath construct at 20 ng/µL. The plasmid-UBI-Cath construct can be as efficient in generating transgene containing the P1 fish, and there may be improved cost efficiency. To the best of our knowledge, this study represents the first description of a targeted exogenous gene insertion in a non-coding region in non-model fish using CRISPR/Cas9 system. Our findings in improving the efficiency of the CRISPR/Cas9 knock-in system may be applicable to other aquaculture species as well.

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8. Appendices

Appendix A Number of channel catfish (*Ictalurus punctatus*) embryos microinjected with different donor DNA constructs and their controls. Treatment groups are dsDNA construct driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath), dsDNA construct driven by carp -actin promoter (dsDNA-BA-Cath) and plasmid DNA with zebrafish ubiquitin promoter (plasmid-UBI-Cath). Four replicates were used for each of the treatment and control groups, injected control (iCTRL) and non-injected control (nCTRL).

Treatment	Donor DNA (ng/μL)	Number of injected embryos				Total
		Trial 1	Trial 2	Trial 3	Trial 4	
dsDNA-UBI-Cath	10	114	70	100	150	434
	20	87	67	100	146	400
	40	89	47	100	150	386
dsDNA-BA-Cath	10	81	64	150	122	417
	20	81	61	150	150	442
	40	121	67	100	100	388
Plasmid-UBI-Cath	10	61	111	100	100	372
	20	66	78	100	100	344
	40	53	56	100	141	350
iCTRL	-	65	36	90	109	300
nCTRL	-	75	54	100	71	300

Appendix B Number of channel catfish (*I. punctatus*) embryos utilized for microinjection and electroporation as transgene delivery strategies and their controls. Treatment groups are dsDNA construct driven by zebrafish ubiquitin promoter (dsDNA-UBI-Cath), dsDNA construct driven by carp β-actin promoter (dsDNA-BA-Cath) and plasmid DNA with zebrafish ubiquitin promoter (plasmid-UBI-Cath). Three replicates were used for each treatment and control, microinjected control (iCTRL), electroporated control (eCTRL) and non-injected/electroporated control (nCTRL).

Treatment	Number of injected embryos			Total
	Trial 1	Trial 2	Trial 3	
Microinjection	80	100	100	280
Single electroporation	104	89	107	300
Double electroporation	90	88	110	288
iCTRL	55	50	56	161
eCTRL	51	50	59	160
nCTRL	50	50	60	160

Appendix C Detailed sequence of dsDNA donor construct driven by zebrafish ubiquitin promoter. Sequences in gray are homologous arms that are part of channel catfish (*Ictalurus punctatus*) chromosome 1; sequences in blue = zebrafish ubiquitin promoter; sequences in green = cathelicidin gene from American alligator; sequences in red = poly-A terminator.

TGCTGGTTCATCTGATTCAAACTCTCTCTATCCTCTGTATGTGTGAGATTTCCTACTGTAGTAC
CTGTGCTGAACTCTGCTCCTTCACTCTCCTGCATCATAACAACATTCCCTGATTCACTGCAGTT
TTTTGGTTTTCCTGATGGTTTTGCAATGTTAATTGTTTCCTTGCTTTCAGTTGTCAGCTTTGCT
GTAGTGTCCTATGCTTTATCATTGTTATGCAGTGCCTGTGAGTGCTCCTGCTGCTGTTACCAGC
AAAGTTCTAGAATTTGTCGAAACATTTATGTTATATATTTCCTGAAAAAAATTCTGAGTAAGTT
CTTAAGTGTTATTGCCAGCAACATAAACAACAGACGGCAAAATGAATAAATGATAACAAAGCAG
TAGGCTTAAATAAACCTAATTTTTATAGGCTGTTCTCTACAACCCTCAAACAGTGATTAGTTTT
GTACTTATAAACTTGCCCTTTCATTCATATTTCAAGAAAATTGGTTCAGAAGATCTGGATATTC
TAGCAGTTGTTCAAGCTCATGGAGGGATCAGTGACCTGATTCCAAATGACTAGGCCTAATCCAG
AAATTAGATGACTGTCAACATAAAAAGGCACAGCACTCACTAGCTGCCCTATATATTTTATTAT
ATTTTACATATATTATTTTATTTATTTAGCTCTGAGTGCTGTACTTTCTGGTTAAAGAAAACTG
CTTACAACAGCTAACCTGTACTACCTCAGGCTCAGGGAATTTGGAACAGGTTTGTCTGGTTTGT
TTCTTTAACCATGCATGCTTGTTTTCAACTATGGCAACACAGTCACATGGGACATTACAGAAAT
GATTTGTCGATGACATGCGACTTTTCTTTAATAAAGCGCAAAGATCCCAAAAAGCAAACTTTTA
ACAAAAATCATATAATTATATTTTCAATCCAGCTTTGTAGCAACTTTGTGCTGCTGTTCACTCA
GCAACAGATAGTCAGTATAAGGTCAGTGTGTCTCAAAGCAGTGCCATCTGTTTCACACATTGCG
TTCTATATATAAGTGTGCTGGTTGACACGACACTGTATAAGGCCTAGGCTAAAACACAAACAAT
GTAGAATGACACTGTGTTTTTTTTGTAAACAAATGTTGTTTTTGGTTAAACATCTTTGTGAAAA
CATCCTCCTGTCATGTATTTGCTATATTCAAATGTTAAACCCGTGCAGAATAGAACATATACAA
AAAAAAACAACACAACACATTTTTAAACATTATTAAATATCAAGTATTGCTGGCAGTTCTGTTT
CTGTTTTACAGTACCCTTTGCCACAGTTCTCCGCTTTTCCTGGTCCAGATTCCACAAGTCTGAT
TCACCAATAGCAAAGCGAATAAACAACCAAAGCAGCCAATCACTGCTTGTAGACTGTCCTGCGA
GACCGGCCCATTCCAGCACATTCTGGAAACTTCCTTTATATGATAATTATAAATACATTTAAAT
TATTGATACAAAACATGTAATTCCTAGAACATAACCATAGCAATCATTAGTTTTCAGGGTAATT
ATGTATTTTTAGGATTTGACTGCGGAAAGATCTGGTCATGTGACGTCTCATGAACGTCACGGCC
CTGGGTTTCTATAAATACAGTAGGACTCTCGACCATCGGCAGATTTTTCGAAGAAGAAGATCAG
TTTCAGGAGCCGTACTGTTCCGTTATGCTATTCAAGAAGCTGAGGAGGAAAATCAAGAAGGGCT
TTAAGAAAATCTTCAAGCGCCTGCCTCCCGTCGGTGTCGGTGTCTCCATCCCACTCGCAGGAAG
GCGGTGAgatccagacatgataagatacattgatgagtttggacaaaccacaactagaatgcag
tgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagct
gcaataaacaagttGTATGGGGTCAGGATCATCCTTAGTGCAGACATTAGGGATAGGGTCACTG
CTAGCGCTATCACTTGTAGCAGCACCGGGTTTGGCGCTAATGCTATCTTCAACATGAGCGGTGT
TTGTGTCAGTGTTAGCATCAGTGCAGGTGTTGGTGTTATTCCTGGGTGCTGCGTTTTTTTCAGT
GTTGCTGTTGTGTTTGTCACTGACTTCTTTCAGCTCTAGCACCGGCACGTCGTAGCTCTGTGAG
TCATTTTC

Appendix D Detailed sequence of dsDNA donor construct driven by common carp β-actin promoter. Sequences in gray are homologous arms that are part of channel catfish (*Ictalurus punctatus*) chromosome 1; sequences in blue = common carp β-actin promoter; sequences in green = cathelicidin gene from American alligator; sequences in red = poly-A terminator.

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TGCTGGTTCATCTGATTCAAACTCTCTCTATCCTCTGTATGTGTGAGATTTCCTACTGTAGTACCTGT
GCTGAACTCTGCTCCTTCACTCTCCTGCATCATAACAACATTCCCTGATTCACTGCAGTTTTTTGGTT
TTCCTGATGGTTTTGCAATGTTAATTGTTTCCTTGCTTTCAGTTGTCAGCTTTGCTGTAGTGTCCTAT
GCTTTATCATTGTTATGCAGTGCCTGTGAGTGCTCCTGCTGCTGTTTTTGATGAAAATCGCTTAGGCC
TTGCTCTTCAAACAATCCAGCTTCTCCTTCTTTCACTCTCAAGTTGCAAGAAGCAAGTGTAGCAATGT
GCACGCGACAGCCGGGTGTGTGACGCTGGACCAATCAGAGCGCAGAGCTCCGAAAGTTTACCTTTTAT
GGCTAGAGCCGGCATCTGCCGTCATATAAAAGAGCGCGCCCAGCGTCTCAGCCTCACTTTGAGCTCCT
CCACACGCAGCTAGTGCGGAATATCATCTGCCTGTAACCCATTCTCTAAAGTCGACAAACCCCCCCAA
ACCTAAGGTGAGTTGATCTTTAAGCTTTTTACATTTTCAGCTCGCATATATCAATTCGAACGTTTAAT
TAGAATGTTTAAATAAAGCTAGATTAAATGATTAGGCTCAGTTACCGGTCTTTTTTTTCTCATTTACG
TGCGAACTCTGCTTAAACTCTAGTTATTCTTTATTAATATGTGGTTATTTTTATATATGTATGTTATC
ATAACTGTACTGGCTATGTCAGGTGGTAATGACTGTAACGTTACGTTACTCGTTGTAGGCACGACATT
GAATGGGCCGGTGTTGAAATAAGTCTTCAACCCCTTTTAACCTCAAAATGTGCTCTGGTTAACAAGGA
TTTTAACAGCTATCAGTATGACTGTGCGGTTTTAAAGCCGTTAGTGAGGCACGTTGCACACTTGATGG
ATGGCCGGAATGGGAAGTTCTTTATGCAGGCAGTGCTGCAGCAGGGTGTGACCTACTTTAGCTAACGT
TAGCCGGCTAACCAGCATTCATCTGCCGGTAACTTGAGTCTAATATTCTCTATGTGATATCGAAGTGA
TCAAAGACACGTCTGTTAGCTCACTTTAACCAACTGTAGTGAAAAATAGCGCAGTGTGCAGCCCTTCA
AGTCTTTCATTTAGGCTGATTATTCAATCATTTTATTAACTATTAACGCGTTACTAAACGTAAGGTAA
CGTAGTCAGTTTTTAATAACTGGTGAAAAGTACTGGTTGGGTTTAAATGGTGACTTATAATTGTGTTG
GAGGGGGAAACCTTTTTGATAAAGGCTATATAATCTCAAATGAATGGGCTGAGGATGGTGTTCACAGG
TGCTTTAGTGAAGTCCGCTCGTGAAGAGTCGCTGAAGTGACTGCAGATCTGTAGCGCATGCGTTTTGG
CAGACGGCCGTTGAAATTCGGTTGAGTAATTGATACCAGGTGAGGCTAGAGGATGTAGAAATTCATTT
GTGTAGAATTTAGGGAGTGGCCTGGCGTGATGAATGTCGAAATCCGTTCCTTTTTACTGAACCCTATG
TCTCTGCTGAGTGCCACACCGCCGGCACAAAGCGTCTCAAACCATTGCCTTTTATGGTAATAATGAGA
ATGCAGAGGGACTTCCTTTGTCTGGCACATCTGAGGCGCGCATTGTCACACTAGCACCCACTAGCGGT
CAGACTGCAGACAAACAGGAAGCTGACTCCACATGGTCACATGCTCACTGAAGTGTTGACTTCCCTGA
CAGCTGTGCACTTTCTAAACCGGTTTTCTCATTCATTTACAGTTCAGCCATGCTATTCAAGAAGCTGA
GGAGGAAAATCAAGAAGGGCTTTAAGAAAATCTTCAAGCGCCTGCCTCCCGTCGGTGTCGGTGTCTCC
ATCCCACTCGCAGGAAGGCGGTGAgatccagacatgataagatacattgatgagtttggacaaaccac
aactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaacca
ttataagctgcaataaacaagttGTATGGGGTCAGGATCATCCTTAGTGCAGACATTAGGGATAGGGT
CACTGCTAGCGCTATCACTTGTAGCAGCACCGGGTTTGGCGCTAATGCTATCTTCAACATGAGCGGTG
TTTGTGTCAGTGTTAGCATCAGTGCAGGTGTTGGTGTTATTCCTGGGTGCTGCGTTTTTTTCAGTGTT
GCTGTTGTGTTTGTCACTGACTTCTTTCAGCTCTAGCACCGGCACGTCGTAGCTCTGTGAGTCATT
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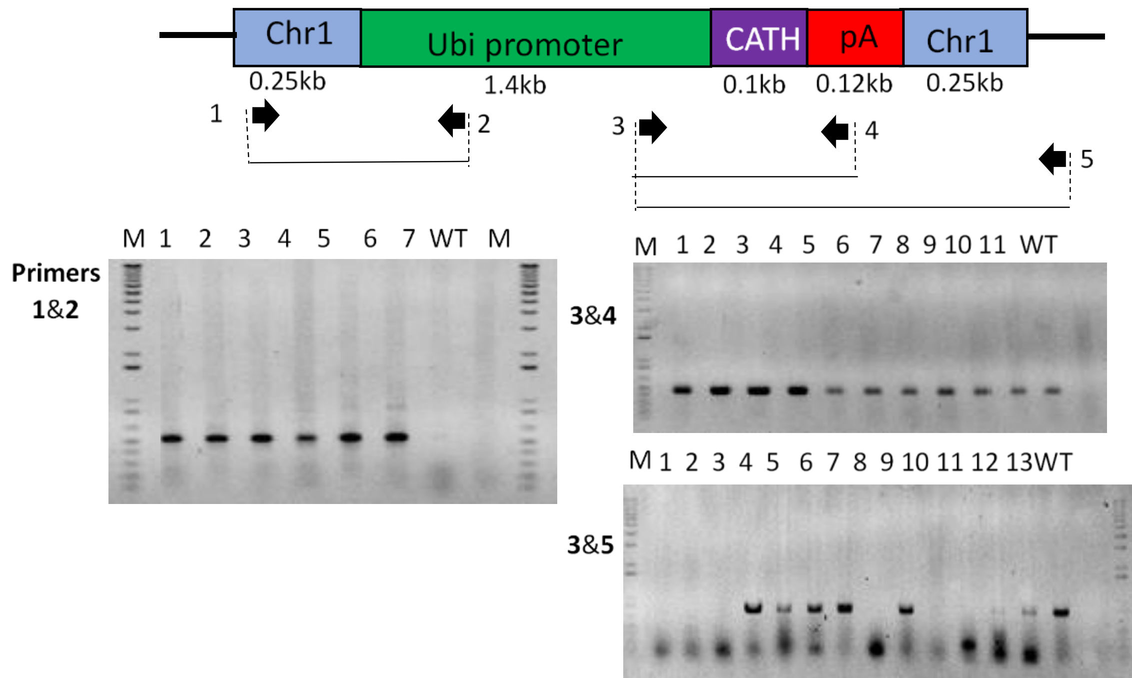
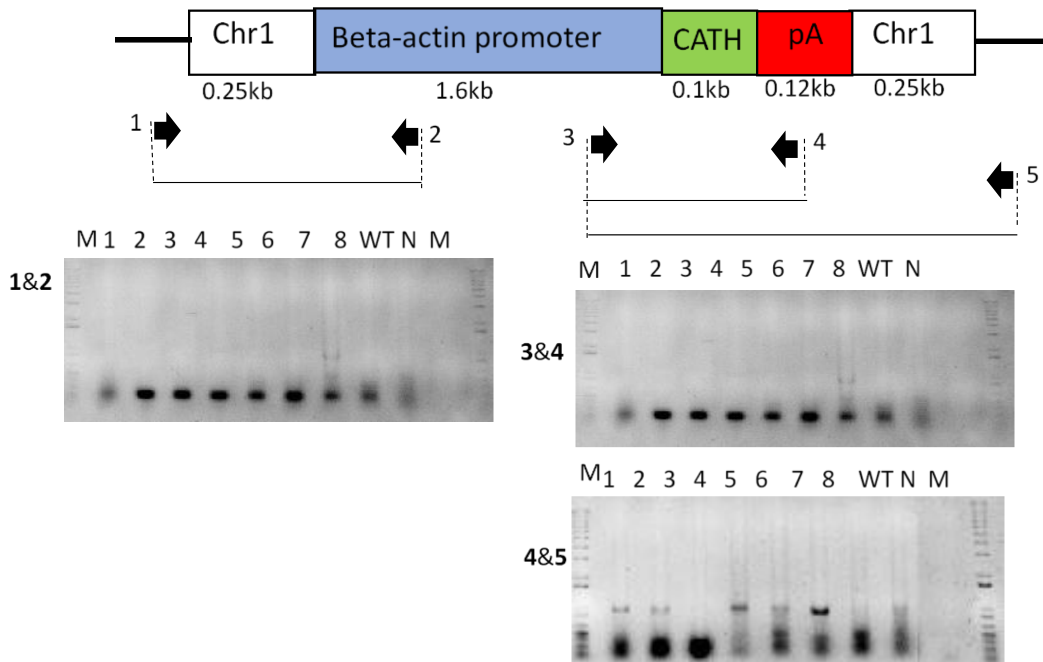
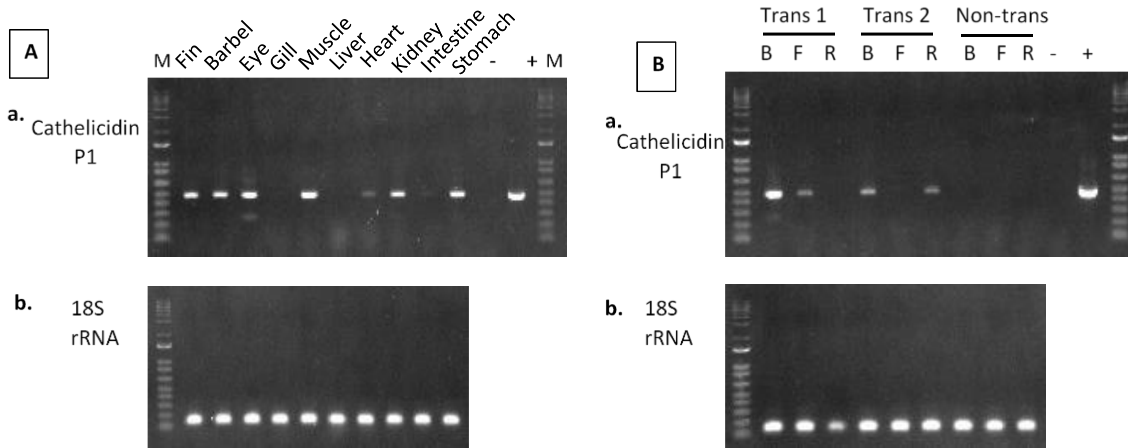
Appendix E Genotyping strategy for CRISPR/Cas9 knock-in of channel catfish (*I. punctatus*) using dsDNA construct driven by zebrafish ubiquitin promoter carrying the cathelicidin gene (dsDNA-UBI-Cath). Gel image of PCR amplification of primer sets at the 5' and 3' junctional regions (primer sets 1&2 and 3&5) and the insert-specific region for cathelicidin gene (primer set 3&4). Numbers in a lane represent individual samples of fish, lane WT represent wild type channel catfish and lane M indicates DNA marker. Presence of a distinct band indicates positive for transgene.



Appendix F Genotyping strategy for CRISPR/Cas9 knock-in of channel catfish (*I. punctatus*) using dsDNA construct driven by β-actin promoter carrying the cathelicidin gene (dsDNA-BA-Cath). Gel image of PCR amplification of primer sets at the 5' and 3' junctional regions (primer sets 1&2 and 3&5) and the insert-specific region for cathelicidin gene (primer set 3&4). Numbers in a lane represent individual samples of fish, lane WT represent wild type channel catfish and lane M indicates DNA marker. Presence of a distinct band indicates positive for transgene.



Appendix G Expression of cathelicidin mRNA in (**A**) different tissues of transgenic channel catfish (*Ictalurus punctatus*) and (**B**) non-invasive tissues, as measured by regular RT-PCR followed by agarose gel electrophoresis. ‘Trans 1’ and ‘Trans 2’ represent P1 transgenic fish while ‘Non-trans’ represents a non-transgenic fish. *B* blood, *F* fin, *R* barbel; +, plasmid DNA positive control; -, PCR reaction in the absence of plasmid DNA. 18S rRNA gene was used as an internal control.



Appendix H Changes in body weights of transgenic, non-transgenic, injected control and non-injected control channel catfish (*Ictalurus punctatus*). Transgenic fish carry the cathelicidin gene inserted into channel catfish genome by CRISPR/Cas9 knock-in system. Body weight was measured at 2 months- and 4 months- post-injection. Mean ± SEM (first rows) and range (second rows) are presented. Means in the same column followed by different superscript letters are significantly different ($P < 0.05$).

Group	N	Weight (g)	
		First Sampling	Second Sampling
Transgenic	36	12.1 ± 0.82^{b}	17.9 ± 0.90^{b}
		5.0 – 20.0	9.5 – 28.0
Non-transgenic	40	13.2 ± 0.79^{b}	18.6 ± 0.88^{b}
		5.0 – 22.5	9.0 – 28.0
Injected control	35	11.8 ± 0.77^{a}	16.7 ± 0.92^{a}
		5.0 – 21.0	9.0 – 29.0
Non-injected control	40	14.3 ± 0.78^{c}	22.6 ± 0.86^{c}
		8.0 – 25.0	14.5 – 32.0